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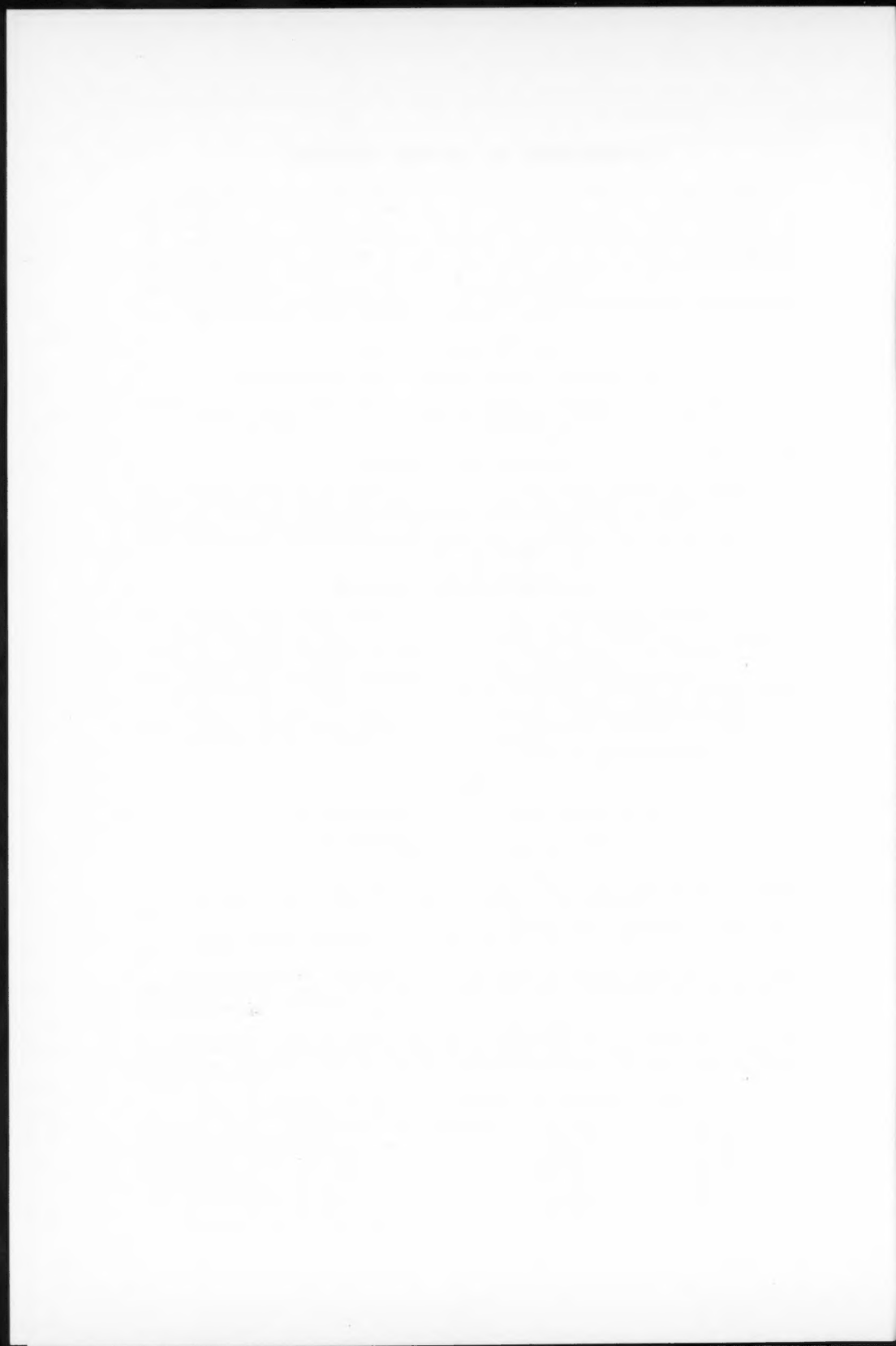
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CORRECTIONS

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Page 21. In Table V, the first column should read as follows:

SE (50%) + synthetic medium
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— synthetic medium
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A PSYCHROPHILIC CRYPTOCOCCUS¹

P.-O. HAGEN AND A. H. ROSE

Abstract

A psychrophilic yeast, isolated from decomposing *Laminaria* found on the beach at Hebron Fjord in Labrador, was shown to be a species of *Cryptococcus*, closely resembling but not identical with *C. mucorugosus* and *C. terricolus*. In a glucose-salts-vitamins medium, the yeast grew most rapidly at 25° C, although the final cell crop was greatest in cultures incubated at 21° C. The yeast grew well at 3° C after a lag period of approximately five days, but no growth was observed in cultures incubated throughout at 30° C or above. Cultures of the yeast which had been incubated at 30° C, but not at 37° C, grew, on being transferred to 16° C, after a lag period that was proportional to the duration of incubation at 30° C. The yeast grew well at 30° C after cultures had been incubated for a period at 16° C, although growth ceased at this higher temperature after a few days; further growth at 30° C could be obtained after the cultures had again been incubated at 16° C. The significance of these results is discussed in relation to the psychrophilic behavior of the yeast.

Introduction

The literature contains numerous reports of microorganisms that are capable of growing well at temperatures (0–20° C) below those normally associated with microbial activity. These microorganisms can be divided into two categories. Some are capable of growth at low temperatures, but grow best in the range 25–40° C. A second category includes organisms that grow best at low temperatures and which are unable or less able to grow in the mesophilic range of temperatures. Terminology is confused with regard to organisms capable of growth at low temperatures, but most workers describe all such organisms as psychrophilic, those in the first category being termed facultative psychrophils, and those in the second category obligate psychrophils.

Psychrophilic strains have been recorded in all of the major groups of microorganisms. Ingraham and Stokes (9), in their review of the literature on psychrophilic bacteria, have reported that these are usually Gram-negative nonsporeforming rods belonging to the genera *Pseudomonas*, *Achromobacter*, and *Flavobacterium*; psychrophilic strains have also been reported among species of *Actinomyces* (6). A number of fungi have also been shown to have a

¹Manuscript received November 29, 1960.

Contribution from the Microbiology Laboratory, Department of Applied Biochemistry, Heriot-Watt College, Edinburgh, Scotland.

psychrophilic habit (4), but only a few psychrophilic yeasts have been reported. Scott (16) isolated a yeast, now called *Candida scottii*, from chilled beef and, more recently, Lawrence, Wilson, and Pederson (10) have reported the isolation from chilled grape juice of other strains of *Candida* that have optimum temperatures for growth in grape juice of approximately 11° C, and which do not grow above 21° C. But, although psychrophilic behavior has been observed in many microorganisms, the biochemical basis of this phenomenon is virtually unknown (9).

This paper describes a psychrophilic species of *Cryptococcus* which was isolated from decomposing *Laminaria* found on the beach at Hebron Fjord in Labrador, and reports on the effect of various incubation temperatures on growth of the yeast.

Materials and Methods

Organism

Isolation of the yeast used in this study is described under Experimental Results. The organism was maintained on slopes of malt wort agar, containing 10% (w/v) of 'Muntona' spray-dried malt extract obtained from Munton & Fison Ltd., Stowmarket, Suffolk, England, and 2% (w/v) agar, and was transferred at monthly intervals. Cultures were stored at 3° C. The methods used for characterizing the yeast were those described by Lodder and Kreger-van Rij (11).

Experimental Cultures

The defined medium of Rose and Nickerson (15) was employed. Tube tests were conducted using 6-ml portions of medium in Samco tubes, covered with anodized aluminum caps obtained from Oxo Ltd., Queen Street Place, London E.C.4 (13). Inocula were prepared by suspending a portion of material from a slope culture of the yeast in 6 ml sterile $M/15$ KH_2PO_4 (pH 4.5). These cells were washed twice with further portions (6 ml) of buffer, and the suspension diluted to contain 1.0×10^7 cells/ml; one drop of this diluted suspension was added to each tube. Cultures were incubated at various temperatures as indicated later. Growth was measured turbidimetrically in the Samco tubes, using the Hilger 'Spekker' absorptiometer, Model H 760, with neutral green-grey H 508 filters and a water blank. Turbidity readings were related to the number of cells/ml using a calibration curve.

Nucleic Acid Estimations

Deoxyribonucleic acid (DNA) estimations were carried out using the procedure described by Ahmad, Rose, and Garg (1). The DNA contents are expressed as the optical density at 260 $m\mu$, in 1 cm cells, of an extract (3 ml) from 30.0×10^7 cells.

Results

Isolation

The yeast was isolated from a portion of decomposing *Laminaria* frond found by Dr. W. J. Nickerson on the beach at Hebron Fjord in Labrador. A piece of partly decomposed blade was placed on the surface of a malt dextrose agar slant, and incubated at laboratory temperature. Several of the organisms

that made up the mixed flora of this culture were subsequently isolated as single colony cultures after repeated plating on malt wort agar. One of these organisms grew as small mucoid colonies on malt wort agar and was shown by microscopic examination to be a yeast. When colonies of this yeast were transferred to liquid malt wort, growth was observed only in cultures incubated at temperatures in the range 3–25° C.

Morphology and Biochemical Characteristics

Colonies of the yeast on malt wort agar were colored off-white and were glistening with a smooth, well-defined edge. Streak cultures on the same medium became tan-colored after several months. Pseudomycelium was not detected in slide cultures of the yeast. No ascospores were seen when the yeast was subcultured on gypsum blocks or on Gorodkova agar.

In cultures of the yeast in liquid malt wort, a slight ring of cells appeared near the surface of the medium, but no pellicle was formed. Cells from 7-day cultures incubated at 16° C were oval or occasionally long-oval, and measured $(3.0\text{--}4.0) \times (5.0\text{--}10.0) \mu$. The cells were surrounded by mucoid capsules, as shown in Indian ink preparations, but the culture fluid did not appear viscous. The capsules gave the characteristic blue color when stained with iodine. No fermentation was detected in liquid cultures using the Durham tube technique.

An examination of the sugar assimilation reactions of the yeast, using the chemically defined medium of Rose and Nickerson (15), indicated that the sugars utilized were glucose, galactose, sucrose, maltose, and lactose. When the ammonium sulphate in the defined medium was replaced by an equal concentration of potassium nitrate, the yeast grew equally well. Filtrates from cultures of the yeast grown in nitrate-containing medium gave a positive reaction when tested for the presence of nitrite. Only very slight growth was observed, however, in a medium in which ethanol (1% (v/v)) was the sole source of carbon.

Mice that had been injected intraperitoneally with a suspension (1.0 ml) containing 1.0×10^7 cells/ml did not develop pathogenic symptoms, and were outwardly normal and healthy for a period of 8 weeks after injection.

Effect of Temperature on Growth

The effect of various incubation temperatures on growth of the yeast is shown in Figs. 1 and 2. The data in Fig. 1 show that, although the final cell crop was greatest in cultures incubated at 21° C, the yeast grew equally rapidly at 25° C. At 3° C, growth was also rapid after a lag period lasting approximately five days. But, at temperatures of 30° C and above, no growth of the yeast was observed. This rapid falloff in growth at temperatures above 25° C is shown in the data in Fig. 2.

Cultures incubated at 30° C immediately after inoculation grew when transferred to 16° C after a lag period that was proportional to the duration of the incubation at 30° C. No growth was observed, however, when cultures that had been incubated at 37° C after inoculation were subsequently transferred to 16° C.

Cultures incubated initially at 16° C grew for a period on being transferred to 30° C, after which growth ceased (Fig. 3). The rate and extent of growth at

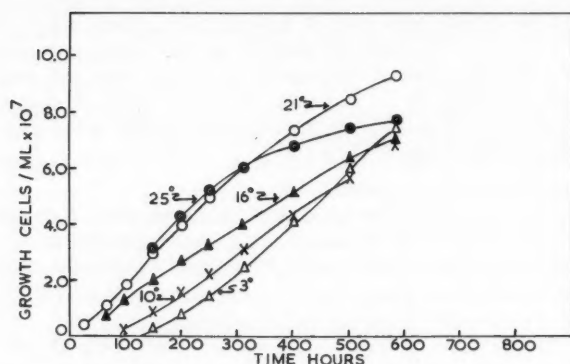


FIG. 1. Growth-time graphs for the cryptococcus growing in a glucose-salts-vitamins medium at various temperatures.

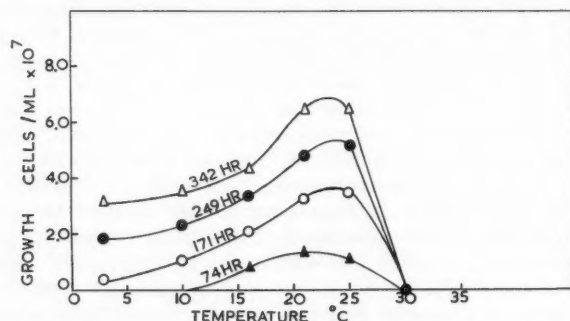


FIG. 2. Growth-temperature graphs for the cryptococcus growing in a glucose-salts-vitamins medium after various periods of incubation.

30° C depended upon the duration of the period of incubation at 16° C. Cultures transferred after 1 day at 16° C grew to only a small extent at the higher temperature, but cultures incubated for 5 days at 16° C grew rapidly at 30° C for a further 5 days before growth ceased. Although not shown in Figs. 3 and 4 this increased rate of growth at 30° C was less marked in cultures that had been transferred from 25° C. When cultures that had stopped growing at 30° C, after being transferred from 16° C, were returned to 16° C, growth resumed after a lag period which was proportional to the duration of the incubation at 30° C; when these cultures were returned to 30° C, they again grew rapidly for a short period (Fig. 4).

Microscopic examination of the cells in cultures that had been subjected to various changes in temperature did not reveal any difference in the average over-all dimensions. Moreover, as shown in the data in Table I, the DNA content of a crop containing 30.0×10^7 cells (measured turbidimetrically) did not vary to any great extent in yeast from cultures that had been grown at

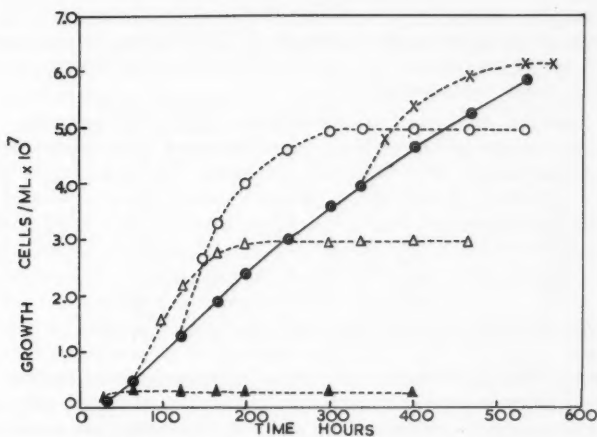


FIG. 3. Effect on growth of the cryptococcus of transferring cultures from 16° C (●) to 30° C after 24 hours (▲), 45 hours (△), 122 hours (○), and 334 hours (×).

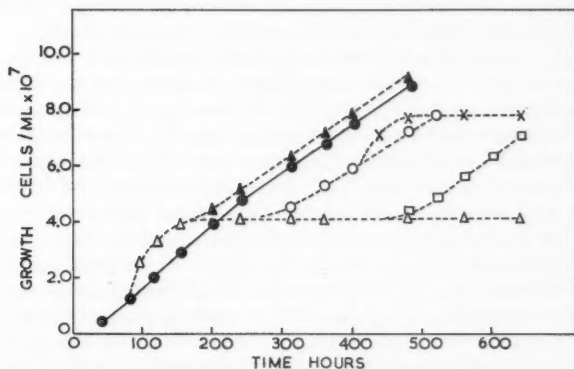


FIG. 4. Effect on growth of the cryptococcus of transferring cultures from 16° C (●) to 30° C after 80 hours (△) and later transferring back to 16° C after 152 hours (▲), 200 hours (○), and 320 hours (□); growth of cultures following a second transfer from 16° C to 30° C after 417 hours is also shown (×).

various temperatures. Since the DNA content of cells is known to remain constant under most conditions, this finding indicated that the relationship turbidity:number of yeast cells did not alter after changes in incubation temperature.

Discussion

Since the yeast described in this paper produced a capsule which stained blue when treated with iodine, and did not produce ascospores or pseudomycelium or ferment sugars, it was placed without hesitation in the genus

TABLE I

Effect of changes in incubation temperature on the DNA content of the cryptococcus

Duration of incubation (hr)				Final growth (cells/ml $\times 10^7$)	DNA content*
16° C	30° C	16° C	30° C		
216	—	—	—	3.15	0.108
408	—	—	—	5.65	0.109
70	146	—	—	1.77	0.100
70	338	—	—	1.77	0.100
70	194	144	—	1.90	0.120
70	194	270	—	3.42	0.112
70	194	240	56	5.23	0.101
70	194	240	72	5.50	0.110

*Optical density at 260 m μ , in 1-cm cuvettes, of extract (3 ml) from 30.0×10^7 cells.

Cryptococcus. Lodder and Kreger-van Rij in their taxonomic treatise described five species of *Cryptococcus* (11), although Benham had previously recognized an additional species, *C. mucorugosus* (3); certain other species of the genus have also been reported since Lodder and Kreger-van Rij published their work. Lodder and Kreger-van Rij, and also Benham, separate species of *Cryptococcus* on the basis of cell shape, pathogenicity, and ability to assimilate nitrate and the sugars glucose, galactose, sucrose, maltose, and lactose. The nonpathogenic nature of the species described herein, together with its ability to assimilate nitrate and each of the above-mentioned sugars, would seem to identify it with *C. mucorugosus*. But cells of *C. mucorugosus* are elongated while those of this newly isolated yeast are more oval. This difference in cell shape was apparent when preparations of the two yeasts were compared; cells of each species were easily distinguishable in vital and fixed preparations containing mixtures of the two yeasts. It would seem, therefore, that the yeast reported in this paper is not identical with any of the species of *Cryptococcus* described by Lodder and Kreger-van Rij (11) or Benham (3). Of the more recently described species of *Cryptococcus*, this yeast bears some resemblance to *C. terricolus*, which was first isolated by Pedersen from Norwegian soil (14). However, cells of *C. terricolus* are reported to contain large quantities of fat, a property which was not observed in the yeast described herein. On the basis of the accepted taxonomic criteria, this yeast would therefore appear to represent a previously undescribed species.

An additional feature which distinguishes this yeast from all other previously described species of *Cryptococcus* is its psychrophilic habit. The ability of the yeast to grow well, after a lag period, at low temperatures (e.g. 3° C), but not at temperatures of 30° C or above, show it to be an obligate psychrophil. Obligate psychrophilic yeasts have been reported previously, but few workers have published data on the effect of various temperatures on growth of these yeasts. However, Lawrence, Wilson, and Pederson (10) reported that the species of *Candida* isolated from grape juice stored at low temperatures grew best in grape juice at around 11° C, while little or no growth was observed at 21° C. By definition, obligate psychrophils are incapable of growth in the mesophilic range of temperatures (e.g. at 30° C), but the effect of these higher temperatures on the viability of these organisms has been little studied. The

finding that cultures of the cryptococcus were still capable of growth after a period of incubation at 30° C suggested that this temperature, although not conducive to growth, did not bring about any irreversible changes in the yeast which rendered it incapable of growth. Incubation at 37° C, on the other hand, would seem to bring about such changes, for cultures that had been incubated at this temperature were incapable of growth on being transferred to 16° C. Although freshly inoculated cultures of the yeast were unable to grow at 30° C, growth at this temperature was observed in cultures that had been incubated previously at 16° C. This would seem to be the first published report of an apparently obligate psychrophil that can be induced to grow at higher temperatures, although similar results have also been obtained with other psychrophilic yeasts in this laboratory (unpublished observations).

Very little is known concerning the biochemical basis of psychrophilic behavior in microorganisms. It has been suggested that, at higher temperatures (e.g. 30° C) but not at lower temperatures, these organisms produce toxic compounds that suppress the action of certain enzymes; but this explanation does not appear very plausible in view of the finding that cultures that had been incubated at 30° C grew well on being transferred to 16° C, unless it is assumed that the inhibitory compounds can only be detoxified at the lower temperatures. Inactivation of mesophilic microorganisms at temperatures above ca. 60° C is usually thought to be a result of the denaturation of enzymes at these higher temperatures; evidence to support this view comes from the finding that some mesophilic microorganisms become more exacting nutritionally at higher temperatures, a result, presumably, of the thermal inactivation of certain synthesizing enzymes (2, 17). It is possible, therefore, that psychrophilic behavior in microorganisms may be a reflection of the thermal inactivation of certain enzymes at comparatively low temperatures of approximately 30° C. The presence of an abnormally thermolabile pantothenate-synthesizing enzyme has been demonstrated in a mutant of *Escherichia coli* (12), while Horowitz and Fling have reported differences between wild strains of *Neurospora* with regard to the thermostability of the enzyme tyrosinase (7). The ability of the cryptococcus to survive at 30° C, but not at 37° C, might therefore be taken to indicate that certain temperature-sensitive enzymes are irreversibly inactivated at 37° C, but reversibly so at 30° C. The ability of the yeast to grow well at 30° C, after an initial period at 16° C, could then be explained by assuming that, at 16° C, the products of the temperature-sensitive reactions accumulate in the cell; on transfer to 30° C, these reserves are utilized but, since they cannot be replenished because of the inactivation of one (or more) of the synthesizing enzymes at this higher temperature, so growth finally ceases. Further incubation at 16° C allows the reserves to be replenished so that they can again be utilized when the cultures are returned to 30° C. The majority of studies on the biochemistry of psychrophilic microorganisms have involved a comparison of the metabolic behavior of a psychrophil with that of a closely related mesophilic species, an experimental approach which has obvious shortcomings (5, 8). But the ability of the cryptococcus to be trained to grow at 30° C provides a more rigorous system whereby the biochemical basis of psychrophilic behavior can be studied, for a comparison of the metabolism of a culture of the yeast growing at a low temperature (e.g.

16° C) with that of a culture which has been transferred from 16° C to 30° C might reveal differences that could give clues as to the nature of the temperature-sensitive metabolic reactions.

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STUDIES ON WESTERN EQUINE ENCEPHALITIS ASSOCIATED WITH WILD DUCKS IN SASKATCHEWAN¹

A. N. BURTON, R. CONNELL, J. G. REMPEL,² and J. B. GOLLOP³

Abstract

It has been shown by many workers in the United States that wild birds are associated with the natural history of Western equine encephalitis (WEE). They have also demonstrated that birds can be infected with WEE virus through the bite of an infected mosquito. Wild ducks of many species make up a large part of the bird population to be found in Saskatchewan from April to November each year. By using the neutralization technique, we have found that WEE antibodies are present in the blood of many wild ducks, indicating previous infection with the virus. Further studies carried out at this laboratory showed that wild ducks can be infected with the virus by the oral route, suggesting another possible means by which birds could be naturally infected.

Introduction

Western equine encephalitis has been a disease of public health importance in Saskatchewan since it was first recognized in 1935 (2). Heavy losses occurred in the horse population during extensive outbreaks in 1938 and 1941, but the disease was not definitely diagnosed in man until 1939 when virus was recovered from two human cases (3). A report issued by the Provincial Department of Public Health in 1941 stated that 543 diagnosed cases of WEE had occurred in man that year, with a mortality rate of 8.1%. Milder outbreaks of the disease have occurred since that time in both the human and the horse population.

Many workers in this field have shown that wild birds and arthropod vectors are associated with the transmission of WEE. Eklund (1) reports that whenever this disease occurs there is an abundant *Culex tarsalis* population and WEE virus is readily isolated from this mosquito species. He also states that present evidence indicates that a cycle of infection between *Culex tarsalis* and wild and domestic birds is an important mechanism for maintaining the virus during the summer months. Kissling *et al.* (5) report that there is little doubt that wild birds serve as the principal source of mosquito infection with Eastern and Western encephalitis viruses during the spring and summer months. However, the reservoir of these viruses through the winter months has not been found. One of the overwintering mechanisms postulated has been a continuing bird-mosquito-bird cycle in the warmer climates throughout the year with seasonal invasion into the more northern areas through the intermediary of migrating birds. Kissling *et al.* (6) isolated WEE virus from wild birds taken in a woodland and cypress swamp area in Louisiana. They also demonstrated the presence of neutralizing antibodies in the blood of many avian species.

Certain mosquito species, namely *Culex tarsalis* (Coquillett), *Culiseta inornata* (Williston), *Aedes dorsalis* (Meigen), *Aedes vexans* (Meigen), and

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Contribution from the Department of Veterinary Science, Biology Department,² University of Saskatchewan, and Canada Wildlife Service,³ Saskatoon, Saskatchewan.

Aedes nigromaculis (Ludlow), suspected or proved to be vectors of the virus, are common in areas of this province where the disease has occurred (8).

Large populations of migratory birds mate and nest in Saskatchewan. During the mid-fifties some 20,000,000 ducks alone returned each spring to the prairie provinces. This represents 65% of North American game ducks, and well over half of these ducks normally breed in Saskatchewan. Mallards and pintails, which make up two-thirds of the population, arrive late in March, and populations of the other 12 species that nest in large numbers on the prairies return during April, with the exception of the blue-winged teal, which usually arrives early in May. Ducks that breed and are reared in this province migrate south from August to November and may be found in the winter throughout the southern United States from the Atlantic and Gulf coasts to the Pacific. Based on Saskatchewan banding records for 12 species, segments of the populations of all of them are known to reach Mexico; some individuals of at least four species (blue-winged teal, shoveller, lesser scaup, and baldpate) get into Central America, the West Indies, and parts of South America.

Since these birds migrate in such vast numbers from areas where WEE has been known to be prevalent, it would seem reasonable to assume that they could introduce the virus into Saskatchewan if they were natural hosts. A study was undertaken at this laboratory to establish that wild ducks are susceptible to the virus in nature, by demonstrating the presence of WEE-neutralizing antibodies in their blood. It was also considered that mosquitoes might not be wholly responsible for the transmission of the virus. Therefore, a further study was carried out in an effort to determine whether these birds could be infected with the virus orally, revealing another possible means by which wild ducks might be naturally infected.

Materials and Methods

Serum-neutralization Tests

Collection of Blood Samples

Wild ducks were trapped, or taken by shooting, in an area of Saskatchewan which harbors many of these birds on their return flight in the early spring. Blood was collected from the wing vein, or by cardiac puncture, using a vacuum tube or hypodermic syringe. During April and May, 1959, 125 blood samples were collected from mature birds, and during April and May of 1960, 471 samples were obtained from ducks of the same age. Serum was removed from the clot as soon as possible, and stored in a mechanical freezer at -20°C until neutralization tests were carried out.

Neutralization Techniques

A human strain of WEE virus (R1), which has been maintained by serial passage in 10-day chicken embryos, was used when neutralization tests were being carried out. The virus has been used in many previous studies undertaken at this laboratory and is still lethal for adult mice when 0.03-ml amounts are given intracerebrally. The LD_{50} titer has remained almost constant between $10^{-8.50}$ and $10^{-9.50}$.

Serial 10-fold dilutions of virus were prepared using 10% inactivated

rabbit serum in beef heart infusion broth as diluent, and constant amounts of undiluted serum were added. The mixtures were incubated for 2 hours at 37° C and 0.1-ml amounts were then inoculated into 10-day chicken embryos by the allantoic route. Screening tests were first done and when a positive or suspicious result was indicated, a further test was carried out to determine the exact titer. Results were recorded approximately 48 hours after embryos were inoculated. In the early part of this work, neutralization tests were done using sera heated at 56° C for 30 minutes. However, none of the sera taken from 471 ducks in 1960 were inactivated by heating before the tests were done. Normal, inactivated rabbit serum was used as a control for the purpose of calculating neutralization indices of the test sera, according to the method described by Reed and Muench (7).

Studies concerning Oral Infection

Experimental Hosts

Wild ducks of several species were included in three groups given virus orally. The birds were held in individual cages. Pre-exposure blood samples from these birds were subjected to a neutralization test and proved negative.

Group 1 consisted of five adult pintails and one adult black duck which received the virus a few days after being captured, while birds in groups 2 and 3 had been trapped when 2-3 weeks of age, and held until they were 2-3 months of age and 6-7 months of age respectively. Many of the birds were of the mallard species, although three pintails, one teal, and one gadwall were included.

Virus Strains Used

WEE virus strain R1 was given to the first group of ducks used for experimental purposes. The other two groups under study received a recently isolated mosquito strain of virus (999-57) supplied by Dr. Leo Thomas, Rocky Mountain Laboratory, Hamilton, Montana. This virus had undergone one chick passage and three baby mouse passages when it was received at this laboratory.

Titration Methods

Virus strains used to infect birds were titered using 10% inactivated rabbit serum in beef heart infusion broth to prepare serial 10-fold dilutions, and 0.1-ml amounts were inoculated by the allantoic route into five 10-day chicken embryos for each dilution. Inoculated embryos were observed over a period of 96 hours. The LD₅₀ titer was then calculated (7).

After birds were given the virus, tissues (including blood) which were taken to determine viral content were examined by the same procedure. All material inoculated into embryos was examined bacteriologically.

Methods of Giving Virus Orally

Water was withheld from six ducks for several hours, after which 300 ml of drinking water, infected with 10⁶ LD₅₀ of R1 virus per ml, was placed before each bird and left for a period of 6 hours (virus was recovered from the water remaining in the vessels at the end of this time). Owing to the fact that ducks waste considerable water when drinking, it is difficult to say how much each bird consumed. By this method, however, it was established that birds

could be infected by the oral route. Further experiments were then carried out in an attempt to measure approximately the smallest amount of virus which would be infective when given orally. A hypodermic syringe with a cannula attached was used to administer definite amounts of virus.

After passing strain 999-57 in 10-day embryos, serial 10-fold dilutions of the virus were prepared to determine the LD₅₀ titer. At the same time nine ducks, 2-3 months of age (group 2), received 1.0-ml amounts of dilutions 10⁻⁶, 10⁻⁷, and 10⁻⁸, using three ducks for each dilution. The LD₅₀ titer of the virus in embryos was 10^{-7.83}.

The same procedure was carried out using a new ampoule of virus strain 999-57 when 12 birds, 6-7 months of age (group 3), were given the virus orally. Dilutions 10⁻⁵, 10⁻⁶, and 10⁻⁷ were administered in 1.0-ml amounts using four birds per dilution and the LD₅₀ titer of the virus proved to be 10^{-8.375}.

Procedure for Determining Infectivity

At definite intervals of time after the ducks had received virus, blood was collected to determine if virus was present and to learn whether WEE-neutralizing antibodies had been produced. Some birds were sacrificed in an attempt to recover virus from internal organs.

Blood samples were examined for the presence of neutralizing antibodies by the method previously described. Blood, taken to examine for viral content, was heparinized and titration tests were carried out using whole blood as the strongest inoculum. When internal organs (brain, lung, liver, kidney, and spleen) were examined for viral content, they were ground in a Waring Blendor with 10% inactivated rabbit serum in beef heart infusion broth to make a 1:10 suspension. Centrifugation was carried out at 2500 r.p.m. for 5-10 minutes and 1000 I.U. of penicillin and 2.5 mg of streptomycin were added per ml of supernatant fluid. Further 10-fold dilutions were then prepared to determine the LD₅₀ titer.

TABLE I

Results of serum-neutralization tests carried out on 125 blood samples collected from adult ducks during April and May, 1959

Species	Number of positive tests	Number of suspicious tests	Number of negative tests
Mallard	13	11	43
Baldpate		2	13
Blue-winged teal		3	3
Pintail	1	8	9
Lesser scaup		2	11
Shoveller	1		2
Redhead			1
Canvasback		1	
Ruddy		1	
	15	28	82
Positive reactors, 12.0%			
Suspicious reactors, 22.4%			

NOTE: A neutralization index below 10 was considered negative, 10 to 49 suspicious, and over 50 positive (4).

Results

Serum-neutralization Tests

The results of tests carried out on 125 blood samples collected from wild ducks during April and May, 1959, and from 471 samples taken during April and May, 1960, are recorded in Tables I and II respectively.

TABLE II

Results of serum-neutralization tests carried out on 471 blood samples collected from adult ducks during April and May, 1960

Species	Number of positive tests	Number of suspicious tests	Number of negative tests
Mallard	32	54	197
Pintail	15	17	77
Baldpate	4	4	46
Blue-winged teal	1		2
Redhead	1		2
Gadwall		1	8
Scaup		1	4
Shoveller		1	3
Canvasback			1
	53	78	340

Positive reactors, 11.25%
Suspicious reactors, 16.55%

NOTE: A neutralization index below 10 was considered negative, 10 to 49 suspicious, and over 50 positive (4).

Studies concerning Oral Infection

Group 1.—Blood was taken from each of the six ducks 24 hours after they were first exposed to the virus in their drinking water, and again 24 hours later (after 48 hours). Virus was recovered from the blood taken from five of the six birds after 24 hours, and from four of the same birds after 48 hours. These ducks were kept to determine whether WEE-neutralizing antibodies would develop in the blood serum. As indicated in Table III, blood samples from all of the birds showed positive reactions when neutralization tests were carried out and antibody titers remained for a prolonged period.

TABLE III

Results of serum-neutralization tests done on blood samples from one adult black duck and five adult pintails

Species and No.	Pre-exposure reaction	Time at which blood was taken after exposure to virus in the drinking water			
		7 days	143 days	238 days	378 days
Black duck	Negative	3.50	3.75	2.75	—
Pintail No. 1	Negative	4.00	3.75	2.75	3.00
Pintail No. 2	Negative	4.75	4.25	3.75	4.50
Pintail No. 3	Negative	4.25	3.00	3.25	—
Pintail No. 4	Negative	5.75	4.50	—	—
Pintail No. 5	Negative	4.50	3.50	4.83	3.75

NOTE: Results are expressed in logarithms of virus neutralized (7). (These indices are based on non-inactivated sera.) Neutralization index log of 2.0 or over is considered positive.

Group 2.—This group consisted of nine ducks, 2–3 months of age, which received measured amounts of virus administered with a hypodermic syringe. The amount of virus given to each bird and the results obtained are outlined in Table IV. Duck No. 1 (group 2) was sacrificed 48 hours after 680 LD₅₀ (0.68×10^3) of virus was administered orally. The bird appeared to be sick at this time. Chick embryo LD₅₀ titers of virus recovered from the tissues were as follows: brain $10^{-1.7}$; lung $10^{-2.5}$; liver $10^{-1.7}$; spleen $10^{-3.4}$; kidney $10^{-1.5}$; and blood $<10^{-1}$ (only two of five embryos inoculated with undiluted blood died). Standard immune sera were used to carry out neutralization tests on virus recovered from this bird and the results were positive. Virus was not recovered from ducks, No. 5 and No. 8, sacrificed after 51 and 52 hours respectively.

TABLE IV

Results obtained when ducks (2–3 months of age) were given measured amounts of WEE virus by the oral route

Duck No.	Dose administered* (LD ₅₀)	Virus recovery from blood after:		Antibody levels† after:	
		24 hr	48 hr	7 days	21 days
1	0.68×10^3	+	+	—	—
2	0.68×10^3	—	—	—	—
3	0.68×10^3	+	+	4.75	5.75
4	0.68×10^2	—	—	—	—
5	0.68×10^2	—	—	—	—
6	0.68×10^2	—	+	4.25	5.50
7	0.68×10^1	—	—	—	—
8	0.68×10^1	—	—	—	—
9	0.68×10^1	—	—	—	—

— Indicates negative findings.

*Total number of chick embryo LD₅₀ of virus administered to each duck.

†Neutralization indices expressed as logarithms of virus neutralized (7). (These results are based on non-inactivated sera.)

TABLE V

Results obtained when ducks (6–7 months of age) were given measured amounts of WEE virus by the oral route

Duck No.	Dose administered* (LD ₅₀)	Titer of virus in† the blood after:		Antibody levels‡ after 14 days
		24 hr	48 hr	
1	2.37×10^4	$10^{-3.0}$	$<10^{-1}$	—
2	2.37×10^4	—	—	—
3	2.37×10^4	$10^{-1.5}$	$<10^{-1}$	3.30
4	2.37×10^4	$10^{-1.025}$	$<10^{-1}$	3.87
5	2.37×10^3	$<10^{-1}$	—	3.75
6	2.37×10^3	$<10^{-1}$	$<10^{-1}$	4.25
7	2.37×10^3	$<10^{-1}$	—	—
8	2.37×10^3	$10^{-1.3}$	$<10^{-1}$	3.09
9	2.37×10^2	$<10^{-1}$	$<10^{-1}$	3.43
10	2.37×10^2	—	—	3.13
11	2.37×10^2	$<10^{-1}$	—	1.88
12	2.37×10^2	$<10^{-1}$	—	—

— Indicates negative findings.

*Total number of chick embryo LD₅₀ of virus administered to each duck.

†Chick embryo LD₅₀ titer.

‡Neutralization indices expressed as logarithms of virus neutralized (7). (The sera were heated at 56° C for 30 minutes.)

Group 3.—These 12 birds, 6–7 months of age, were also given a measured amount of virus with a hypodermic syringe. The amount of virus given to each bird and the results obtained are outlined in Table V. Duck No. 1 (group 3) was sacrificed 52 hours after receiving virus. Chick embryo LD₅₀ titers of virus recovered were as follows: lung 10^{-2.17}; spleen 10^{-1.0}; liver <10⁻¹. Neutralization tests were carried out on virus recovered from each of these tissues. Rabbit and duck antisera were used for the tests. The results showed that virus was present in the lung, liver, and spleen of this bird.

Discussion

Prior to our recent work, there had been no attempt made to associate WEE with wild birds in Saskatchewan. Results of serum-neutralization tests established that there are antibodies present in the blood of many wild ducks. These findings indicate that the birds were infected either through the bite of an infected mosquito, or in a manner still unknown. Since numerous workers in this field have shown that WEE virus can be transmitted through the bite of an infected mosquito, there is no doubt that this is one method by which birds acquire the infection. However, it is quite possible that this is not the only method. From our studies it has been experimentally proved that wild ducks are readily infected by the oral route. It would appear that a latent infection is produced when the virus is given in this manner, although on one occasion a young bird was observed to be very sick. It was sacrificed, and virus was recovered from the tissues (lung, liver, spleen, brain, and kidney).

Ducks, which were given access to drinking water containing a relatively high concentration of virus (R1), were all infected as demonstrated by the presence of neutralizing antibodies in the blood serum. It was also shown that WEE antibodies can remain in the blood for more than a year.

When ducks, 2–3 months of age, were given a measured amount of virus (999-57) orally, it was found that 680 LD₅₀ (0.68×10^3) produced infection in two out of three birds, while 68 LD₅₀ (0.68×10^2) produced infection in only one of three birds.

Later, when ducks in group 3 were given measured amounts of virus, recovery was made from the blood, or neutralizing antibodies were demonstrated in the blood serum of 11 of the 12 birds. Since neutralizing antibodies were present in the blood of three of the birds which received the smallest amount of virus (2.37×10^2 or 237 LD₅₀), and virus was recovered from the blood of the other bird which received this amount, it is quite likely that an even smaller dose would have produced positive results. Of interest is the fact that there were two birds in this group (receiving different amounts of virus) which failed to produce neutralizing antibodies, although virus was recovered from the blood 24 hours after it had been administered; while antibodies were present in the blood serum of still another bird, yet virus was not recovered from the blood.

Attempts to recover virus from tissues (including blood) were usually carried out within the 48-hour period after the birds were given the virus since it is during this time, in our experience, that virus is most likely to be present.

It is quite possible that virus strain 999-57, recovered from mosquitoes, was avian in origin and, therefore, readily adaptable to this species.

Although only wild ducks were used throughout these studies, our findings suggest another possible means by which wild birds could be naturally infected with the WEE virus.

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INTERSUBGROUP AND INTRASUBGROUP ANTIGENIC RELATIONSHIPS WITHIN THE GENUS SHIGELLA: SUBGROUP A¹

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Abstract

Intersubgroup antigenic relationships between the O antigens of cultures of *Shigella dysenteriae* 2 and 10 (subgroup A) and those of strains of *Shigella boydii* 1 (subgroup C) and between the O antigens of cultures of *S. dysenteriae* 8 and those strains of *S. boydii* 15 were reported. Also, several previously reported intra- and inter-subgroup relationships were confirmed and further elucidated. The relationships were important in definitive typing of the bacteria.

The genus *Shigella* is divided into four subgroups or species on the basis of the biochemical and serological reactions of the bacteria contained in each. These subgroups are A, *Shigella dysenteriae*; B, *Shigella flexneri*; C, *Shigella boydii*; and D, *Shigella sonnei*. The serotypes within each subgroup are numbered consecutively with Arabic numerals.

A few O antigenic relationships between serotypes of *Shigella* that belong to different subgroups previously have been described. For example, an O antigenic relationship between cultures of *S. dysenteriae* 2 and *S. boydii* 15 was mentioned by Ewing, Hucks, and Taylor (5) and by Ewing, Reavis, and Davis (8); and Ewing *et al.* (7, 8) called attention to an important reciprocal relationship between the O antigens of strains of *S. dysenteriae* 3 and those of serotype 3615-53, the status of which is provisional.

The purpose of this paper is to direct attention to relationships of the O antigens of cultures of *S. dysenteriae* 2 and *S. dysenteriae* 10 and those of strains of *S. boydii* 1, which apparently have not been reported, and to attempt to elucidate further several intrasubgroup antigenic relationships within *Shigella* subgroup A.

The antisera employed were prepared with cultures collected from many different parts of the world. A few of the older antisera, prepared 15 to 20 years ago, were made by injection of unheated, formalinized broth cultures. However, the majority were O antisera that were prepared by the injection of broth cultures that had been heated at 100° C for 2 hours, or, as was the case with more recently produced O antisera, by injection of antigens prepared according to the method of Roschka (9). Ten *S. dysenteriae* 2, four *S. dysenteriae* 8, six *S. boydii* 1, four *S. boydii* 4 antisera, and two or more O antisera for each of the other serotypes listed in the tables were employed in the study. Also, numerous strains that belonged to each serotype were included. The methods used in the preparation of O antigen suspensions for both slide and tube agglutination tests, as well as for absorption of antisera, were the same as those previously described (2, 4, 5, 7).

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The results of tube and slide agglutination tests with the *Shigella* serotypes under investigation are given in Tables I and II, and a summary of the intrasubgroup O antigenic relationships of *Shigella* of subgroup A is given in Table III.

Attention is directed to the intersubgroup relationship between the O antigens of *S. dysenteriae* 2 and *S. boydii* 1. This relationship has been studied since about 1953 by the senior author and has been demonstrated in all lots of antiserum prepared with these serotypes. The titers of strains of *S. dysenteriae* 2 in six *S. boydii* 1 antisera were 1:80 to 1:1280 and *S. boydii* 1 cultures were agglutinated by 10 *S. dysenteriae* 2 antisera to titers of 1:80 to 1:2560. The homologous titer of these antisera were 1:2560 to 1:20,480. However, the extent of the cross agglutination reactions did not appear to be directly correlated with the homologous titers of the antisera in all instances. For example, the titer of *S. boydii* 1 antigen suspensions in one *S. dysenteriae* 2 antiserum having a homologous titer of 1:2560 was 1:640, while in another *S. dysenteriae* 2 antiserum with a homologous titer of 1:10,240, the titer of *S. boydii* 1 antigens was 1:320. The possibility that a variety of form variation might be operative in cultures of these shigellae is being investigated. While these relationships appeared to be relatively minor, as judged by the titers obtained in some of the antisera, they nevertheless were apparent in slide agglutination tests in unabsorbed antisera in dilutions of 1:5 or 1:10. Therefore, the relationships are of importance in exact serological typing of shigellae. Further, it should be noted that absorption of *S. boydii* 1 antiserum with suspensions of *S. boydii* 4, or *S. boydii* 4 plus *Alkalescens*-Dispar O group 1, did not remove agglutinin for *S. dysenteriae* 2 (Tables I, II).

Dr. K. P. Carpenter of the International Shigella Center (London) reported (personal communication, 1957) that strains of *S. dysenteriae* 8 were divisible into two subserotypes and that the relationship extant between them was of the *a*, *b-a*, *c*³ variety. These observations were confirmed in this laboratory and the results of reciprocal agglutinin absorption tests with the two subserotypes of *S. dysenteriae* 8 are given in Tables I and II. The O antigens of cultures of *S. dysenteriae* 2 did not appear to be significantly related to those of either subserotype of *S. dysenteriae* 8. However, an intersubgroup relationship was noted between the O antigens of *S. dysenteriae* 8a, 8c, and *S. boydii* 15, and absorption of *S. boydii* 15 antiserum with a suspension of *S. dysenteriae* 8a, 8c reduced the titer of this antiserum for strains of *S. dysenteriae* 2 from 1:640 to 1:80 (Table I).

A reciprocal relationship between the O antigens of *S. dysenteriae* 10 and those of *S. boydii* 1 (Tables I and II) also was noted some time ago and has been studied at intervals. Absorption of antisera for *S. dysenteriae* 10 with suspensions of *S. boydii* 1 reduced the titers of the antisera for strains of *S. dysenteriae* 2 but did not remove all agglutinin for this serotype (Tables I and II). Similarly, absorption of *S. boydii* 1 antisera with suspensions of *S. dysenteriae* 10 reduced the titers of the antisera for *S. dysenteriae* 2 but did not remove all agglutinin for it. Thus, it was apparent that in the relationship

³These designations are used to characterize the relationships and should not be considered as permanent designations for the antigens involved.

TABLE I
Results of tube agglutination tests with unabsorbed and absorbed antisera

O antisera	Absorbed by:	O antigen suspensions (100 C, 1 hour)						
		A2*	A8a,8b	A8a,8c	A10	C1	C4	C15
A2*		10,240†	0		640	640	0	640
A2	A10	2,560			0	160		160
A2	C1	2,560			0	0		320
A2	C15	5,120			0	320		0
A8a,8b		0	20,480	10,240	0	0	0	320
A8a,8b	A8a,8c		2,560	0				0
A8a,8b	C15		10,240	10,240				0
A8a,8c		0	1,280	10,240	0	0	0	40
A8a,8c	A8a,8b		0	2,560				40
A8a,8c	C15		640	10,240				0
A10		640	0	0	2,560	640	40	0
A10	A2	0			640	40		
A10	C1	160			640	0		
C1		640	40	0		5,120	640	0
C1	A2	0			320	5,120	640	
C1	A10	160			40	5,120	640	
C1	C4	160			0	5,120	0	
C1	C4 + A-DO1	80				2,560	0	
C4		0					10,240	
C4	C1					640	5,120	
C4	C1 + A-DO1					0	2,560	
C15		640	40	320	0	0	0	20,480
C15	A2	0	40	160				2,560
C15	A8a,8b	640	0	160				2,560
C15	A8a,8c	80	0	0				2,560

*These designations are merely a convenient way of labeling cultures. The capital letter stands for the subgroup to which a strain belongs and the number is the serotype designation within the subgroup. Thus, A2 is *S. dysenteriae* 2; C1 is *S. boydii* 1, etc. A-DO1 is Alkaliescens-Dispar O1 (*Escherichia coli* O group 1). †Figures indicate the highest dilution that gave strong agglutination. Zero indicates no reaction at lowest dilution tested (1:40).

between *S. dysenteriae* 2 and *S. dysenteriae* 10 and *S. boydii* 1, two or more factors were operative.

TABLE II
Results of slide agglutination tests with unabsorbed and absorbed antisera

O antisera	Absorbed by:	O antigen suspensions (100 C, 1 hour)						
		A2*	A8a,8b	A8a,8c	A10	C1	C4	C15
A2*		4†	—	—	3s	4	—	4
A2	A10	4	—	—	—	2s	—	3
A2	C1	4	—	—	—	—	—	4
A2	C15	4	—	—	—	4	—	—
A8a,8b		1	4	4	—	—	—	4s
A8a,8b	A8a,8c	—	4	—	—	—	—	± to 1
A8a,8b	C15	—	4	4	—	—	—	—
A8a,8c		—	4	4	—	—	—	4s
A8a,8c	A8a,8b	—	—	4	—	—	—	2 to 4
A8a,8c	C15	—	4	4	—	—	—	—
A10		4	—	—	4	4	—	—
A10	A2	—	—	—	4	—	—	—
A10	C1	2s	—	—	4	—	—	—
C1		4	—	—	4s	4	4	—
C1	A2	—	—	—	— to 1s	4	4	—
C1	A10	2s	—	—	—	4	4	—
C1	C4	4	—	—	—	4	—	—
C1	C4 + A-DO1	3	—	—	—	4	—	—
C4		—	—	—	—	4	4	—
C4	C1	—	—	—	—	—	4	—
C4	C1 + A-DO1	—	—	—	—	—	4	—
C15		4	—	4s	—	—	—	4
C15	A2	—	—	—	—	—	—	4
C15	A8a,8b	4	—	2	—	—	—	4
C15	A8a,8c	—	—	—	—	—	—	4

*See note, Table I.

†Figures denote degree of agglutination reaction: 4, 4+ complete, rapid reaction; s, slowly.

The relationship between the O antigens of *S. dysenteriae* 2 and those of *S. dysenteriae* 10 previously reported by Ewing *et al.* (5, 8) was reconfirmed, as was the relationship of *S. dysenteriae* 2 and *S. boydii* 15 cultures (Tables I and II). Although several cultures that belonged to each of the serotypes within subgroups A and C were tested, no other intersubgroup relationships of significance between members of these subgroups were noted during the study.

The manner in which most of the antisera and all of the antigens used in the above-mentioned studies were prepared precluded any possibility that alpha, beta, or fimbrial antigens might be involved in the relationships described. Further, fimbrial antigens are not known to occur in cultures of shigellae other than *S. flexneri* serotypes 1 to 5 inclusive (1, 3).

On the basis of the results reported (Tables I and II) absorbed antisera are recommended for use in the differentiation of the serotypes of *Shigella*

TABLE III
Intrasubgroup antigenic relationships of *Shigella* subgroup A (*S. dysenteriae*)

O antisera (unabsorbed)	O antigen suspensions (100 C, 1 hour)										
	A1	A2	A3	A4	A5	A6	A7	A8a,8b	A8a,8c	A9	A10
A1*	10,240†	0	0	0	0	0	0	0	0	0	0
A2	0	10,240	0	0	0	0	0	0	40	0	640
A3	0	0	20,480	0	0	0	0	0	0	0	0
A4	0	0	0	10,240	0	0	0	0	0	0	0
A5	0	0	0	0	5,120	0	0	0	0	0	0
A6	0	0	0	0	0	10,240	0	0	0	0	0
A7	0	0	0	0	0	0	5,120	0	0	0	0
A8a,8b	0	0	0	0	0	0	0	20,240	10,240	0	0
A8a,8c	0	0	0	0	0	0	0	1,280	10,240	0	0
A9	0	0	0	0	0	0	0	0	0	5,120	0
A10	0	640	0	0	0	0	0	0	0	0	2,560

*.†See Table I.

mentioned. These absorbed antisera are (see Table I for meaning of abbreviations):

A2	absorbed by C1 and C15	A10	absorbed by A2
A8a,8b or 8a,8c	absorbed by C15	C1	absorbed by A2, C4, and A-D01
A8a,8b	absorbed by A8a,8c	A10	absorbed by C1 and A-D01
A8a,8c	absorbed by A8a,8b	C15	absorbed by A2 and A8a,8c

A summary of intrasubgroup relationships of cultures of *S. dysenteriae* is given in Table III. In this table the homologous titers listed were average and were representative of those obtained with several lots of antisera and with antigen suspensions prepared with a number of strains of each serotype. Two unilateral reactions occurred in certain antisera. Cultures of *S. dysenteriae* 2 were agglutinated by two of three antisera for *S. dysenteriae* 6 in dilutions of 1:80 to 1:320, but strains of *S. dysenteriae* 6 were not agglutinated by any of 10 antisera for *S. dysenteriae* 2. Similarly, suspensions of *S. dysenteriae* 3 reacted (1:80 to 1:320) in four of seven antisera for *S. dysenteriae* 5, but seven antisera for the former serotype failed to react with cultures of the latter. Since these reactions did not occur in all antisera and were unilateral, they were not recorded in Table III.

No relationships have been noted between the K antigens of the aforementioned serotypes of *Shigella* although additional strains of the various subgroup A and C serotypes must be examined before a definite statement can be made in this regard. However, an interesting variation has been noted in the K antigens (see 6) of *S. dysenteriae* 2, which is being investigated further. Preliminary investigations indicated that the K antigens of this serotype apparently segregated into two varieties or forms that can be differentiated serologically. One of these was shared by cultures of *Escherichia coli* O112a, 112c, while the other was not.

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β -D-1,2-GLUCANASES IN FUNGI¹

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Abstract

β -D-1,2-Glucans apparently occur rarely in nature, and yet many fungi have the ability to produce a β -1,2-glucanase capable of hydrolyzing them. The β -1,2-glucanases in fungi are adaptive, like the β -1,4-glucanases, and thus differ from the β -1,3- and the β -1,6-glucanases which are constitutive. Enzymatic hydrolysis of the β -1,2-glucan is random, a series of β -1,2-linked oligosaccharides, including sophorose, being formed.

Introduction

As part of a study of microbial polysaccharases (14, 15), sources of the hitherto unstudied β -1,2-glucanases have been investigated. The availability of specific glucanases should assist materially in the elucidation of polysaccharide structure (8, 12) and aid in the selective removal of individual polysaccharides from mixtures.

β -1,2-Glucanases* are of particular interest in view of the possibility of their utilization to obtain sophorose (β -D-glucosyl-(1 \rightarrow 2)-D-glucose) by enzymatic hydrolysis of the β -D-1,2-glucan of the crown gall organism, *Agrobacterium tumefaciens*. Sophorose is a highly active inducer of the cellulase of *Trichoderma viride* (7).

Methods

A. Preparation of β -1,2-Glucan

β -1,2-Glucan, admixed with a glucan gum, was produced as described by McIntire (9) by growing *Agrobacterium tumefaciens* (Wisconsin A-6) on a 2% sucrose medium. After removal of cells and concentration of the culture fluid, the gum was removed by precipitation with ethanol (2 vol.). After further concentration of the supernatant, the β -1,2-glucan was precipitated with ethanol (9 vol.). The fact that this glucan is β -1,2 linked was shown by Putnam *et al.* (13). Our material is of the same nature. On enzymatic hydrolysis it gave sophorose as the disaccharide product.

B. Production of β -1,2-Glucanase

The organisms were grown on glucose (0.3%) in a basal medium containing (per liter): KH_2PO_4 2.0 g; $(\text{NH}_4)_2\text{SO}_4$ 1.4 g; urea 0.3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.3 g; CaCl_2 0.3 g; and trace elements (Fe 1.0 mg; Mn 0.5 mg; Co 0.5 mg; Zn 0.8 mg). Since the enzyme is adaptive in most fungi, β -1,2-glucan had to be added to the medium. Because of the scarcity of this substrate, the amount used was kept low (0.05%). As an alternate procedure, the *Agrobacterium tumefaciens* culture fluid, centrifuged to remove cells, and containing both gum and glucan (and sometimes unconsumed sucrose), was added to an equal volume of basal medium. The cultures (50 ml/250 ml flask; pH 6.0) were grown on a reciprocal

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*This abbreviated name for the enzyme will be used hereafter, in place of β -D-1,2-glucanase.

shaker (90 strokes/minute) at 29° C. The medium was occasionally modified by incorporation of small amounts (0.05%) of nutrient broth (Baltimore Biological Laboratories) or of proteose peptone (Difco).

C. Estimation of β -1,2-Glucanase Activity

Enzyme solution (1/2 ml) was added to an equal volume of β -1,2-glucan solution (0.4% in 0.05 M citrate buffer pH 4.5) and incubated at 50° C for 2 hours. Reducing sugars produced as a result of hydrolysis were determined by the dinitrosalicylic acid (DNS) method of Sumner and Somers (16). A solution has one β -1,2-glucanase unit per ml if, when assayed as above, it produces 0.4 mg reducing sugar as glucose.

D. Electrophoretic Separations

The electrophoretic separations were carried out according to procedures developed by Miller *et al.* (10). A concentrated enzyme solution (0.1 ml) was applied to the center of a 100 cm long starch-paste block. After 20 hours at 4.0° C, 0.1 ionic strength phosphate, pH 7.0, 8 volts per cm, the block was cut into 1-cm pieces and each portion extracted with 6 ml of water. Enzyme determinations (5) were made on the extracts by incubation with the appropriate substrate: β -1,2-glucan (β -glucosidase included in assay), β -1,3-glucan (= laminaran), salicin, and cellobiose. The substrate for β -1,6-glucanase was pustulan isolated from *Umbilicaria pustulata* (4).

Results

A. Preparation of β -1,2-Glucan

While the original investigators (2, 9) reported yields of β -1,2-glucan as high as 3.0 g/liter, we have never been able to achieve this. A considerable effort has been made, without success, to modify the conditions of growth so as to increase the yield. As previously reported (9), enriching the medium to favor growth usually leads to decreased yields of glucan. The addition of manganese leads to increased gum formation.

The consumption of sugar frequently stops short of completion. Processing of these cultures has led to products containing reducing sugars, because of the high ethanol concentrations required for precipitation of the glucan. Such preparations have been used in the growth experiments, but only sugar-free β -1,2-glucan has been used in the direct assay of β -1,2-glucanase.

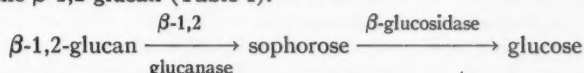
Our best β -1,2-glucan preparation on complete acid hydrolysis (N H₂SO₄ for 2 hours at 100°) gave 88% glucose. On partial acid hydrolysis (0.33 N H₂SO₄ for 1 hour at 100°) the presence of glucose and sophorose was indicated by paper chromatography using the solvent system propan-2-ol:acetic acid:water (67:10:23, by volume). In addition, the partial acid hydrolyzate stimulated cellulase production in *T. viride*.

The nature of the gum is not known. It is primarily a glucan (1) but the partial acid hydrolyzate does not induce cellulase and therefore is probably free of sophorose. The gum differs also from the β -1,2-glucan in that it is resistant to the action of the β -1,2-glucanases.

B. Detection of β -1,2-Glucanases

Sophorose and higher dextrans, because of the linkage at carbon-2, do not

reduce DNS. In the presence of β -glucosidase, the sophorose produced by β -1,2-glucanase action is hydrolyzed to glucose. β -Glucosidases alone have no action on the β -1,2-glucan (Table I).



Most of the β -1,2-glucanase preparations contain β -glucosidase but the level is too low to convert all of the sophorose produced into glucose (Table I). For detection of β -1,2-glucanases in preparations deficient in β -glucosidase it is necessary to add some from another source. Either the β -glucosidase of almond emulsin or that of *Aspergillus luchuensis* is suitable.

TABLE I
Effect of addition of β -glucosidase to β -1,2-glucanase assay

β -1,2-Glucanase source	QM No.	Activity as glucose (mg/ml)		
		Plus buffer	Plus β -glucosidase of:	
			<i>A. luchuensis</i>	Emulsin
<i>Absidia ramosa</i>	3256	.20	.25	.25
<i>Aspergillus fumigatus</i>	45h	.06	.28	.23
<i>A. quadricinctus</i>	6874	.48	.60	.63
<i>A. sydowi</i>	31c	.06	.11	.00
<i>Fusarium oxysporum</i>				
v. <i>bulbigenum</i>	6213	.10	.12	.14
<i>Penicillium brefeldianum</i>	1873	.27	.31	.36
<i>P. funiculosum</i>	474	.16	.32	.36
<i>P. quadrilineatum</i>	7871	.03	.13	.11
Buffer		0	.01	.02

C. Search for β -1,2-Glucanases

Over 200 organisms have been tested for their ability to produce β -1,2-glucanase on a medium containing the β -1,2-glucan. Since negative results may indicate that our growth conditions were not satisfactory, enumeration of these is not included here. Of the organisms tested, over two-thirds produced either a trace, or none, of the enzyme. None of the bacteria (nine species), *Streptomyces* (five cultures), or yeasts (two species) was active.

Of the active organisms (Table II), many fall into closely related taxonomic groups. All members of the *Penicillium funiculosum* series were active (*P. funiculosum*, five strains; *P. varians*, *P. islandicum*, *P. verruculosum*). All members of the *Aspergillus fumigatus* group were active (*A. fumigatus*, *A. aureolus*, *A. auratus*, *A. fischeri*, *A. quadricinctus*). But only one-half of those in the *P. javanicum* series were active (*P. brefeldianum*, *P. queenslandicum*, *P. parvum*, *P. quadrilineatum*). All fusaria were active, but varied greatly from test to test.

The yields of β -1,2-glucanase are low (Table II) when compared to the yields of the other polysaccharases (β -1,4-glucanase = cellulase, β -1,3-glucanase, β -1,6-glucanase, and α -glucanase) we have worked with (6, 14, 15). As with cellulase, β -1,2-glucanase is an adaptive enzyme in most fungi. When grown on a medium containing both glucose and glucan, it does not appear

until after the glucose has been consumed. In most instances it is liberated slowly, and reaches a maximum concentration in the medium after 20 days. On much longer incubation, the activity gradually decreases, the rate of inactivation varying from one organism to another.

In addition to the above screening program, 31 carbohydrase preparations (commercial, and our own) were tested and found inactive. These include α - and β -glucosidases, β -1,4-glucanase, β -1,3-glucanase, α -1,4-glucanase, fructanase, chitinase, and xylanase.

TABLE II
Yields of β -1,2-glucanase by some of the most active fungi

Organism	β -1,2-Glucanase	
	QM	μ /ml. (max.)
<i>Aspergillus auratus</i>	7861	2.0
<i>A. aureolus</i>	1906	2.0
<i>A. quadricinctus</i>	6874	8.0
<i>A. sydowi</i>	31c	0.2
<i>A. unguis</i>	8f	4.0
<i>Beauveria bassiana</i>	7438	1.0
<i>Fusarium oxysporum</i>	6213	3.0
"	21c	3.0
"	47e	0.8
" " <i>v. cubense</i>	7694	3.0
<i>Penicillium brefeldianum</i>	1873	2.0
<i>P. funiculosum</i>	391	0.8
<i>P. funiculosum</i>	7563	3.0
<i>P. melinii</i>	1931	18.0
<i>P. parvum</i>	1878	7.0
<i>P. quadrilineatum</i>	7871	6.0
<i>P. verruculosum</i>	3698	6.0

TABLE III
Carbohydrase activities of β -1,2-glucanase preparations

Organism	QM No.	β -Glucanase, μ /mg				β -Glucosidase (cellobiase)
		β -1,2-	β -1,3-	β -1,4-	β -1,6-	
<i>Aspergillus auratus</i>	7861	1.8*	1.3*	0.1*	NT	NT
<i>A. quadricinctus</i>	6874	2.0	11.0	0	0.3	1.2
<i>A. unguis</i>	8f	1.7	0.8	0	0.3	0.8
<i>Beauveria bassiana</i>	7438	0.9*	0.4*	0*	NT	NT
<i>Fusarium oxysporum</i>	21c	1.1*	1.0*	0*	NT	NT
<i>F. oxysporum v. cubense</i>	7694	1.1	0.6	0	0	0.8
<i>Penicillium brefeldianum</i>	1873	0.7	0.7	0	0.4	1.1
<i>P. funiculosum</i>	391	1.1	0.9	0	0.1	0.9
<i>P. funiculosum</i>	7563	0.3	1.3	0	0	1.3
<i>P. melinii</i>	1931	8.5	1.5	0	3.6	0.5
<i>P. parvum</i>	1878	9.0	26.0	0	9.0	2.3
<i>P. quadrilineatum</i>	7871	1.4	0.6	0	1.2	0.3
<i>P. verruculosum</i>	3698	2.8	4.0	0.1	0.1	1.9

*Based on μ /ml rather than on μ /mg.

D. β -1,2-Glucanases: Properties

1. Effect of Temperature on Activity (Fig. 1)

As is well recognized, the optimum temperature is a function of duration of the experiment, and inactivation of enzyme counterbalances an increase in rate. For 30 minute incubation, the activity rises over the tested range (40–60° C) for all five preparations used (curves are shown only for *P. melinii*). For the 60-minute incubation, 60° C was again the optimum, though 50° C was as good for two preparations. It is obvious that in these two, inactivation was playing a role. A temperature of 50° C appears to be suitable for most experiments of short duration.

2. Effect of pH on Activity (Fig. 1)

The optimum pH is about 4.0 (± 0.4) for enzymes of all six species tested. Representative curves are shown for *P. melinii* and for *P. funiculosum*. At pH 7.0, the activity is very low. On the acid side of the optimum, there is greater variability. At pH 2.8, the activity is less than 25% of that at the optimum for four of the species, but it is relatively higher for the other two.

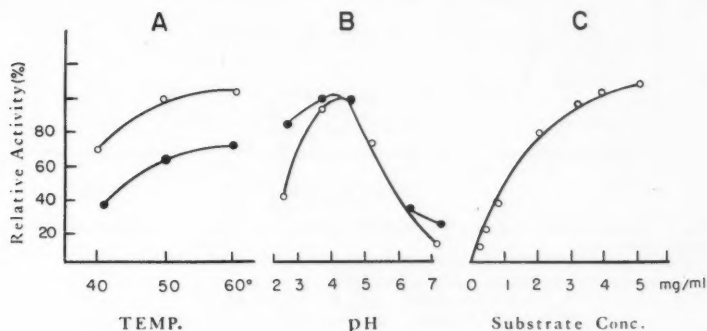


FIG. 1. β -1,2-Glucanase.

A. Effect of temperature on β -1,2-glucanase activity of *P. melinii*; \circ 60 minutes, \bullet 30 minutes, pH 5.3.

B. Effect of pH on β -1,2-glucanase activity; \circ *P. melinii*, \bullet *P. funiculosum* QM 391, temp. 50° C.

C. Effect of substrate concentration on β -1,2-glucanase activity. Enzyme of *P. melinii* (40° C; pH 4.0).

3. Effect of Substrate Concentration on Activity (Fig. 1)

Enzymatic activity increases as the substrate concentration increases to 4 mg/ml. Assay solutions should, therefore, contain about 5 mg/ml, rather than the 2 mg/ml that we have been using because of our limited supply of substrate.

E. Presence of Other Carbohydrases in the β -1,2-Glucanase Preparations (Table III; Fig. 2)

β -1,2-Glucanase in fungi is an adaptive enzyme, and is not found under conditions favoring the production of other adaptive enzymes such as cellulase. However, the constitutive enzymes do appear with the β -1,2-glucanase. So,

while it is easy to find other glucanases free of β -1,2-glucanase, it is not easy to find β -1,2-glucanase free of β -1,3-glucanase or β -1,6-glucanase. In addition, the oligosaccharase, cellobiase, is invariably present.

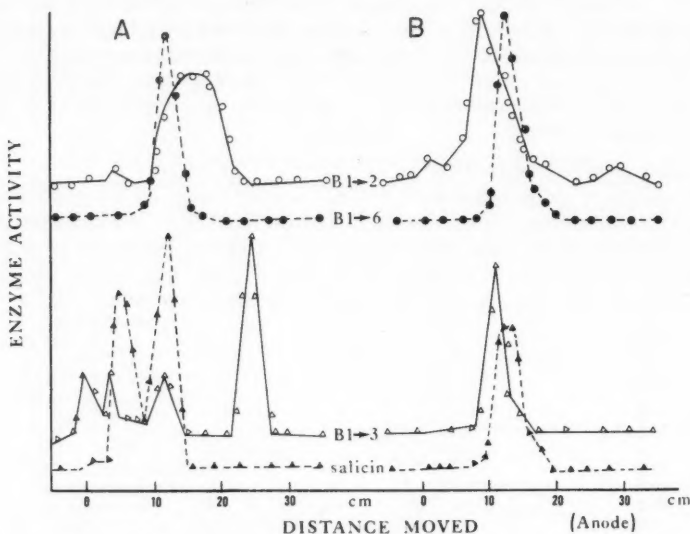


FIG. 2. Electrophoretic separation of β -1,2-glucanase from other glucanases and from β -glucosidase.

A. Enzymes of *Penicillium melinii* QM 1931.

B. Enzymes of *Penicillium parvum* QM 1878.

○ β -1,2-Glucanase. △ β -1,3-Glucanase.

● β -1,6-Glucanase. ▲ β -Glucosidase (vs. salicin).

Most of the points in the regions of no activity have been omitted.

The β -glucanases can be separated from each other by zone electrophoresis (5). Electrophoretic patterns (Fig. 2) indicate that each β -glucanase is a distinct enzyme. The preparations used were free of β -1,4-glucanase (cellulase) activity. In the preparation from *P. parvum*, the β -1,2-glucanase moved 9 cm, the β -1,3-glucanase 11 cm, and the β -1,6-glucanase 12 cm towards the anode. Salicinase (β -glucosidase) moved 13 cm. All of these activities thus appear to be separate, despite considerable overlapping. In *P. melinii*, the β -1,2-glucanase is a broad peak, probably representing several components, that moved 14–19 cm towards the anode. The β -1,6-glucanase is a single peak at 12 cm. The β -1,3-glucanase has a major peak that moved 25 cm and three distinct minor peaks, one of which (12 cm) coincides with the single β -1,6-glucanase peak, with one salicinase peak, and with cellobiase (not shown). Another (at 4 cm) coincides with the single amylase peak (not shown). The β -glucosidase of *P. melinii* has two components (5, 12 cm) active against the β -glucoside, salicin, but only one of these (at 12 cm) has the ability to hydrolyze cellobiose (curve not shown). Perhaps the best separated components are the

β -1,2-glucanase at 18 cm, and the β -1,3-glucanase peak at 25 cm. These are free of the other enzymes tested for.

F. Enzymatic Hydrolysis Products of β -1,2-Glucan

The β -1,2-glucanase preparations act on β -1,2-glucan to produce glucose, sophorose, and higher members of the β -1,2 series (Fig. 3). Because of the presence of β -glucosidase, glucose predominates in the hydrolyzates. In order to promote the accumulation of sophorose, Δ -gluconolactone was tested as an inhibitor of the β -glucosidases of ten β -1,2-glucanase preparations. It was found to give 50% inhibition of the hydrolysis of glucan to glucose at concentrations of 0.01 to 0.1 that of the glucan. Addition of gluconolactone to the reaction mixture effectively reduced the production of glucose from sophorose (Fig. 3), without affecting the hydrolysis of β -1,2-glucan to sophorose and other intermediates. The sophorose produced was isolated from the reaction mixture by carbon column chromatography, and crystallized from methanol/ethanol. The melting point was 191–192° C.

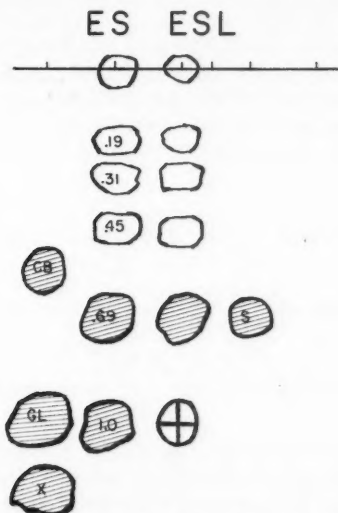


FIG. 3. Chromatogram of enzymatic hydrolyzate of β -1,2-glucan. Enzyme of *P. funiculosus* QM 391; 50° C, pH 4.0, 30 minutes.

ES = Enzyme + substrate.

ESL = Enzyme + substrate + Δ -gluconolactone.

Numbers = R_F values.

CB = cellobiose, S = sophorose, GL = glucose, X = xylose.

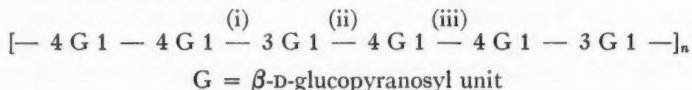
○ = Readily apparent on chromatogram. ⊕ = Visible only under ultraviolet.

Discussion

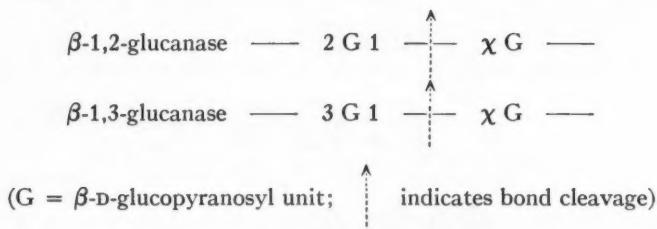
Fungal β -1,2-glucanases are adaptive enzymes which catalyze the hydrolysis of a polysaccharide found only in a few species of *Agrobacterium*. Kooiman (3) attributed the very slight hydrolysis of the β -1,2-glucan by a heat-resistant cellulase of *Myrothecium verrucaria* (9 days at 35° C) to the cellulase itself.

While we cannot disprove this interpretation, our experience has been that each improvement in technique strengthens the view that each linkage type is hydrolyzed by a different enzyme.

The β -glucanases have a high degree of specificity, the nature of which is just becoming clear through the work of Parrish and Perlin (11). These workers have used two different enzyme preparations, a β -1,3-glucanase and a β -1,4-glucanase, to degrade a β -D-glucan containing mixed linkages.



It was found that the β -1,4-glucanase hydrolyzed linkage (iii) preferentially while the β -1,3-glucanase hydrolyzed linkage (ii) preferentially. Cleavage of linkage (i) was not observed with either enzyme. Thus, the nature of the linkage cleaved, i.e., (β -1,3) or (β -1,4), is less important a factor than the *position of substitution* of the glucosidic unit which is attacked. In agreement with this new concept, our results (unreported) on a glucan of mixed linkage (lichenan) show that the β -1,4-glucanases in general produce cellobiose (i.e., with the β -1,4 link intact) indicating cleavage of β -1,3 bonds. The β -1,3-glucanases, acting on the same substrate, yield laminaribiose (and no cellobiose) indicating cleavage of β -1,4 bonds. Designation of glucanases (as β -1,2, etc.) is meant to imply that the enzyme hydrolyzes the glucosidic bond of a glucose substituted at the designated position:



Awareness of this new concept is important to those wishing to interpret the enzymatic hydrolysis of polysaccharides of mixed linkage types. It is for this reason that we introduce this material into the present paper.

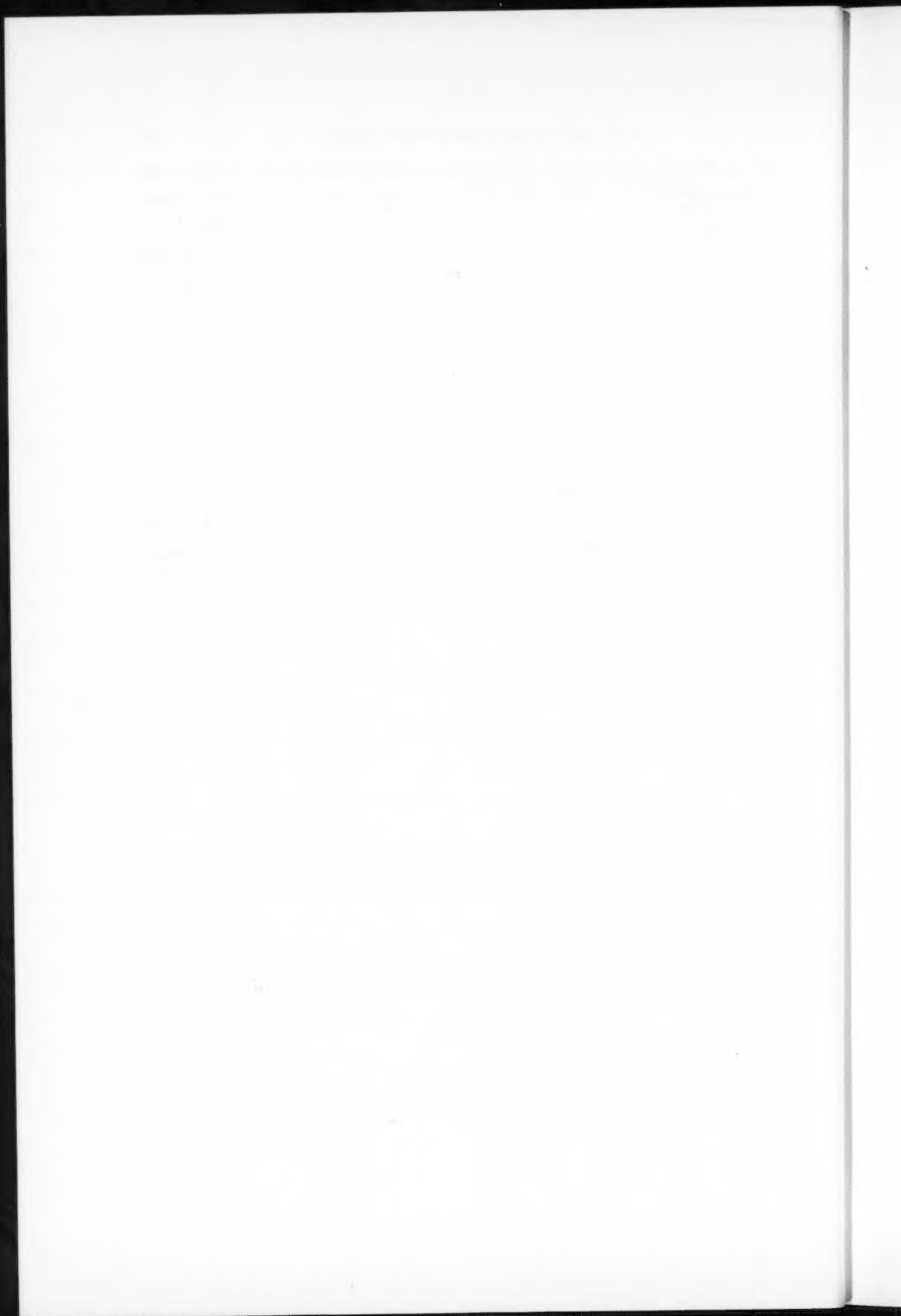
Acknowledgments

Fungus cultures were supplied by E. Simmons from the Quartermaster Culture Collection; the *Agrobacterium tumefaciens* culture, from A. Hildebrandt (University of Wisconsin). The electrophoretic separations were made under the guidance of G. L. Miller.

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THE OCCURRENCE OF TWO DIFFERENT GLUTAMIC DEHYDROGENASES IN *NEUROSPORA*¹

B. D. SANWAL AND MADHU LATA²

Abstract

Many wild-type strains of *Neurospora crassa* produce two glutamic dehydrogenases, one specific for triphosphopyridine nucleotide and another for diphosphopyridine nucleotide. The enzymes have been separated from one another and purified about 50-fold. Michaelis constants have been reported for various substrates. The pH optima of the DPN- and TPN-specific enzymes are 8.3 and 7.5 respectively.

The possible reasons for the presence of two different enzymes catalyzing the same reaction are discussed.

Introduction

The glutamic dehydrogenase of *Neurospora crassa* has been investigated by Fincham and co-workers in a series of papers (2, 3, 4, and 5) primarily from the genetical point of view. The enzyme has been reported by these authors to be specific for triphosphopyridine nucleotide (TPN). In single-gene amination-deficient mutants (*am*⁻), the enzyme was found to be either lacking or present in suboptimal quantities (3). The mutants, apparently lacking the enzyme, could grow only with preformed sources of α -amino acids in the medium. Nicholas and Mabey (10) later demonstrated a glutamic dehydrogenase in certain wild-type strains of *N. crassa*, which, like the liver enzyme (12), could use both reduced diphosphopyridine nucleotide (DPNH) and reduced triphosphopyridine nucleotide (TPNH) in the reductive amination assay of glutamic dehydrogenase.

In the fungi so far studied, glutamic dehydrogenase is either DPN- or TPN-specific or, as in yeast, two separate glutamic dehydrogenases are known to occur, one specific for DPN and another for TPN (7). Sanwal (13) has also demonstrated two separate glutamic dehydrogenases in *Fusarium*. In view of the conflicting reports obtained with *Neurospora*, it was likely that here also two separate enzymes were involved, one specific for DPN and another for TPN. Besides this interest in the glutamic dehydrogenase of *Neurospora* from the point of view of coenzyme requirement, our interest was primarily in the one gene—one enzyme hypothesis. If one-gene amination-deficient mutants could not grow in the absence of glutamic acid or other suitable sources of α -amino nitrogen, did it mean that one-gene mutation has affected—if proved—two separate enzymes? Our preliminary experiments in this direction lent strong support for the suspicion that there were two distinct and separate glutamic dehydrogenases in *Neurospora* with different coenzyme requirements.

This report describes the isolation, purification, and some properties of the

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two glutamic dehydrogenases of *N. crassa*, wild-type strain R⁺, kindly supplied by Dr. P. K. Issac of the Botany Department of the University of Manitoba.

Materials and Methods

Inoculum

The fungus was grown in Difco *Neurospora* agar slants for 4 days. The conidia were lightly scraped off from the top of the tubes and suspended in deionized water. Two milliliters of a heavy suspension of the conidia was used to inoculate 200 ml medium in 1-liter Erlenmeyer flasks.

Medium and Growth Conditions

Liquid medium N of Vogel (15) was used in all experiments. The cultures were grown in a shaking machine operating at 50 strokes per minute at 28° C for 48 hours.

Preparation of Cell-free Extracts

The mycelium, after 48 hours' growth, was harvested on two layers of muslin, washed repeatedly with distilled water, and excess water squeezed off. The nearly dry mass of cells was frozen at -20° C for 2-3 hours before use. All the operations after this step were conducted at 0-3° C. The frozen cells were mixed with twice their weight of acid-washed levigated alumina and rubbed vigorously in a mortar till the whole mass acquired a 'sticky' consistency (2-5 minutes). The disrupted cells were extracted with 3 volumes of a buffer containing 0.1 M Tris (tris-(hydroxymethyl)aminomethane) and 5×10^{-4} M β -mercaptoethanol, pH 8.5, and the alumina and cell-wall debris removed by centrifugation at 11,000 g for 15 minutes. The opalescent supernate was used for assay and subsequent purification.

Reagents

DPN, TPN, and enzymatically reduced DPNH and TPNH were obtained from Sigma Chemical Company, St. Louis, Missouri. γ -aluminum hydroxide gel was prepared according to the method of Willstätter and Kraut (17).

Enzyme Assays

Both TPN- and DPN-specific enzymes were assayed by following the disappearance of the appropriate coenzyme in the reductive amination assay of α -ketoglutarate at 340 m μ .

1. *TPN-specific enzyme*.—The assay system consisted of 20 μ moles α -ketoglutarate, 120 μ moles ammonium sulphate, 0.40 μ moles TPNH, suitably diluted enzyme preparation, and 273 μ moles Tris in a final volume of 3.0 ml. The pH of the mixture was 7.5. Quartz cuvettes of 1-cm light path were used and reaction was carried out at 22-24° C.

2. *DPN-specific enzyme*.—The assay system consisted of 20 μ moles α -ketoglutarate, 120 μ moles ammonium sulphate, 0.46 μ moles DPNH, suitably diluted enzyme preparation, and 273 μ moles Tris in a final volume of 3.0 ml. The pH of the reaction mixture was 8.4. The mixture was prepared in quartz cuvettes of 1-cm light path and reaction was carried out at 22-24° C.

In both of the above cases reference cuvettes were used with all the components of the assay mixture except α -ketoglutarate. The reaction was

started by the addition of enzyme and the decrease in optical density was measured every 15 seconds for 60 seconds. The rate of the reaction was linear for approximately 1 minute. The first two 15-second readings were used to calculate the activity of the enzymes. One unit of the enzyme is defined as the amount which causes a decrease of 0.001 in optical density in 1 minute. Specific activity is defined as the number of units per mg protein in the enzyme preparation. Protein was determined by the spectrophotometric method of Warburg and Christian (16).

Experiments and Results

Purification of TPN-specific Glutamic Dehydrogenase

The pH of the crude cell-free extract was adjusted to 6.8 with 10% acetic acid and the precipitate removed by centrifugation at 11,000 *g* for 10 minutes. The supernatant fluid was subjected to heat treatment in order to inactivate the DPN-specific enzyme present in the extracts. The protein concentration was first adjusted to 6–7 mg per ml with 0.1 *M* Tris, pH 7.0, and the enzyme extract brought quickly (1–2 minutes) to 53° C in a water bath and held at this temperature for 5 minutes. After rapid cooling, the precipitate was centrifuged off at 11,000 *g*. To the supernatant solution solid ammonium sulphate was added to 0.40 saturation and after it was stirred for 15 minutes, the heavy precipitate was discarded by centrifugation. More solid ammonium sulphate was added to bring the solution to 0.55 saturation and it was stirred for 20 minutes. The precipitate was recovered by centrifugation at 19,000 *g* for 10 minutes and dissolved in a small volume of 0.02 *M* phosphate buffer containing 5×10^{-4} *M* β -mercaptoethanol, pH 6.5. Protein concentration at this stage was adjusted to 1.5–2.0 mg per ml. The solution was dialyzed for 8 hours against the same buffer with frequent changes.

The dialyzed solution was fractionated with *C*_γ-aluminum hydroxide according to the procedure of Ochoa *et al.* (11). Gel was added at the rate of 0.2 mg per mg protein and the suspension was stirred for 30 minutes. The gel was recovered by centrifugation at 3000 *g* for 10 minutes, washed once with distilled water, and eluted for over 30 minutes with 0.5 volume of 0.2 *M* phosphate buffer containing 5×10^{-4} β -mercaptoethanol, pH 8.4. More gel was added to the supernate at the rate of 0.2 mg per mg protein and treated in the same way. The process was repeated till all the enzyme had

TABLE I
Procedure for the purification of TPN-linked glutamic dehydrogenase

Step	Total volume, ml	Protein, mg	Total units	Specific activity	Purification
1. Crude extract	60	2,621	142,000	54	—
2. Heat treatment	60	358	98,400	275	5.0
3. Ammonium sulphate 0.4–0.55 saturation	20	41	58,000	1,450	26.8
4. <i>C</i> _γ -alumina treatment					
1st eluate	5	3.75	6,000	1,600	—
2nd eluate	5	3.50	9,200	2,630	48.6
3rd eluate	5	3.70	5,500	1,490	—

been adsorbed. The fractions showing the highest specific activity were pooled, lyophilized, and stored at -20° if not directly used. The enzyme can be kept at this temperature without loss of activity for about 7 days. An outline of the purification procedure is given in Table I.

Purification of the DPN-specific Glutamic Dehydrogenase

The pH of the cell-free extract was adjusted to 7.0 with 10% acetic acid and the residue, obtained after centrifugation at 11,000 g for 15 minutes, was discarded. Solid ammonium sulphate was added to the supernatant solution to 0.35 saturation and the precipitate again discarded after centrifugation. The ammonium sulphate concentration was raised to 0.45 saturation and the precipitate allowed to form for 30 minutes. It was recovered by centrifugation at 19,000 g for 10 minutes and dissolved in a small volume of 0.02 M phosphate buffer containing 5×10^{-4} M β -mercaptoethanol, pH 6.5. The enzyme preparation was dialyzed against the same buffer for 4 hours with frequent changes. The protein concentration of the solution was adjusted to 4–5 mg per ml and it was fractionated stepwise by C_{γ} -aluminum hydroxide gel in the same way as the TPN-specific enzyme. Protein was eluted every time after a preliminary distilled water wash with 0.5 volume of 0.2 M phosphate, 5×10^{-4} M β -mercaptoethanol, pH 8.4. Nearly all the DPN-specific enzyme is adsorbed in the first two treatments. These first two eluates showing the highest specific activity were pooled and the protein concentration adjusted to 0.5–1.0 mg per ml with 0.2 M phosphate buffer, pH 8.4.

To the combined fractions from the last step ammoniacal ammonium sulphate (5 ml concentrated NH_4OH in 100 ml 0°C saturated ammonium sulphate) solution was added with constant stirring to 0.35 saturation. The small precipitate formed after 1 hour was recovered by centrifugation at 19,000 g for 15 minutes and dissolved in a small convenient volume of 0.02 M Tris, pH 7.5. The solution was dialyzed for 6 hours against the same buffer and when not directly used, was lyophilized, and stored at -20°C for 24 hours. The enzyme proved to be very labile in a purified state and could not be stabilized either by adding inert proteins or raising the ionic strength of the buffer. Under the above experimental conditions, the DPN-specific enzyme can be completely separated from the TPN-specific enzyme. The outlines of the purification procedure are given in Table II.

TABLE II
Procedure for the purification of DPN-linked glutamic dehydrogenase

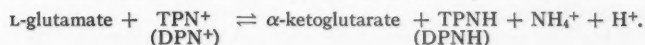
Step	Total volume, ml	Protein, mg	Total units	Specific activity	Purification	s.a. DPN s.a. TPN
1. Crude extract	35	616	30,800	50	—	1.04
2. Ammonium sulphate, 0.35–0.45 saturation	10	43	18,000	419	8.4	21.0
3. C_{γ} -alumina treatment, 1st and 2nd eluate	10	7.7	13,000	1,680	33.6	—
4. Ammoniacal ammonium sulphate, 0.35 saturation	5	2.0	7,400	3,700	74.0	—

pH Optima

The DPN-specific enzyme has a pH optimum of 8.3 and the TPN-specific enzyme 7.5 (Fig. 1).

Products of the Reaction

Both TPN- and DPN-specific enzymes catalyze the reaction:



The equilibrium of the reaction favors the formation of glutamate. In the forward direction, the formation of α -ketoglutarate was demonstrated by chromatography of the 2,4-dinitrophenylhydrazone derivative (1). The system in the forward direction consisted of 3 μ moles TPN (or DPN), 720 μ moles L-glutamate, 980 μ moles K_2HPO_4 , 5 mg TPN-specific enzyme of specific activity 2600 (or 4 mg DPN-specific enzyme of specific activity 3600) in a volume of 5.0 ml. The final pH was 7.5 (or 8.3 for the DPN-specific enzyme) and the incubation time was 30 minutes at 30° C. In the reverse direction, formation of glutamic acid was demonstrated in a system consisting of: 200 μ moles α -ketoglutarate, 2.0 μ moles TPNH (or DPNH), 1.13 mmoles ammonium sulphate, 2 mg TPN-specific enzyme of specific activity 2600 (or 1.5 mg DPN-specific enzyme of specific activity 3600), 980 μ moles K_2HPO_4 in a final volume of 5.0 ml. The pH of the mixture was 7.5 for the TPN-specific enzyme and 8.3 for the DPN-specific one. The mixture was incubated

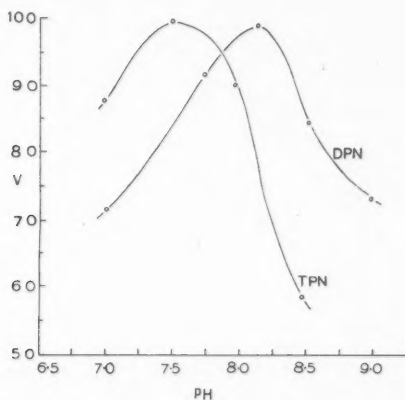


FIG. 1. The pH optima of the DPN-specific and TPN-specific glutamic dehydrogenases.

Assay system for DPN-specific enzyme: $L=1$ cm; total volume 3.0 ml; $T=22-24^\circ\text{C}$; $\lambda=340$ m μ . Each cuvette contained 0.5 mg DPNH, 0.06 ml saturated ammonium sulphate, 0.2 ml 0.2 M α -ketoglutarate, 0.02 ml purified enzyme, made up with 0.2 M phosphate buffer to 3.0 ml.

Assay system for TPN-specific enzyme: $L=1$ cm; total volume=3.0 ml; $\lambda=340$ m μ ; $T=22-24^\circ\text{C}$. Each cuvette contained 0.3 mg TPNH, 0.06 ml saturated ammonium sulphate, 0.2 ml 0.2 M α -ketoglutarate, 0.02 ml purified enzyme, made up with 0.2 M phosphate buffer to 3.0 ml.

Ordinate = V = percentage of activity at pH 7.5 for the TPN-specific enzyme and pH 8.3 for the DPN-specific enzyme. Abscissa = pH value.

for 45 minutes at 30° C. At the end of this period 5 ml ethanol was added to the reaction mixture and it was evaporated to dryness by means of a jet of air at 50° C. The residue was extracted with 0.2 ml ethanol and cochromatographed with an authentic sample of L-glutamic acid. The spot was developed by ninhydrin spray.

Substrate Specificity

DPN is ineffective as an electron acceptor in the deamination of L-glutamic acid by the TPN-specific enzyme and TPN is ineffective for the DPN-specific enzyme. D-Glutamic acid, L-glutamine, L-aspartic acid, L-asparagine, L-alanine, L-valine, L-proline, L-threonine are ineffective for both enzymes in the forward direction. DPNH cannot be substituted for TPNH in the reductive amination assay of the TPN-specific enzyme and TPNH in the assay of the DPNH enzyme. For both enzymes, α -ketobutyric acid, α -ketoisocaproic acid, α -ketoisovaleric acid cannot be replaced for α -ketoglutarate in the reductive amination.

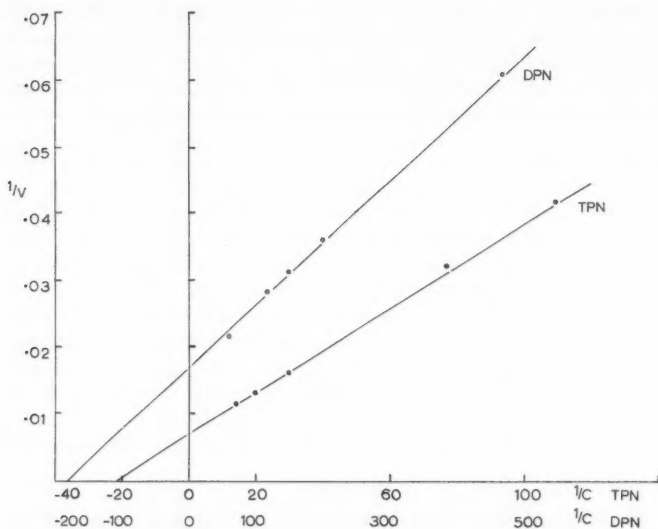


FIG. 2. Rate of the oxidative deamination of L-glutamic acid as a function of the concentration of L-glutamic acid with the DPN-specific and the TPN-specific glutamic dehydrogenases.

DPN-specific enzyme: $L=1$ cm; $T=22-24^{\circ}$ C; $\lambda=340$ m μ . Each cuvette contained 1.0 mg DPN, sodium glutamate (conc. in the figure), 0.7 mg purified enzyme, made up to 3.0 ml with 0.1 M Tris, pH 8.3.

TPN-specific enzyme: $L=1$ cm; $T=22-24^{\circ}$ C; $\lambda=340$ m μ . Each cuvette contained 0.8 mg TPN, sodium glutamate (conc. in the figure), 1.2 mg purified enzyme, volume made up to 3.0 ml with 0.1 M Tris, pH 7.5.

Ordinate, $1/V=1/\text{O.D. change} \times 1000$ per minute; abscissa, $1/C=1/\text{mole L-glutamate per liter}$.

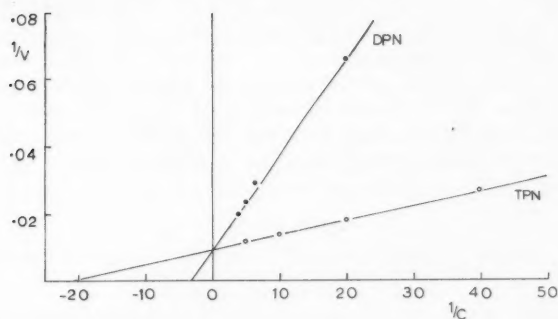


FIG. 3. Rate of the oxidative deamination of *L*-glutamic acid as a function of the concentration of oxidized DPN and TPN with the DPN-specific and the TPN-specific glutamic dehydrogenases.

DPN-specific enzyme: $L=1$ cm; $T=22-24^{\circ}\text{C}$; $\lambda=340\text{ m}\mu$. Each cuvette contained 240 μmoles sodium glutamate, oxidized DPN (concentration in the figure), 0.7 mg purified enzyme, made up to 3.0 ml with 0.1 *M* Tris, pH 8.3.

TPN-specific enzyme: $L=1$ cm; $T=22-24^{\circ}\text{C}$; $\lambda=340\text{ m}\mu$. Each cuvette with 240 μmoles sodium glutamate, oxidized TPN (concentration in the figure), 1.2 mg purified enzyme and 0.1 *M* Tris, pH 7.5 to 3.0 ml.

Ordinate, $1/V=1/\text{extinction difference} \times 10^3$ per minute; abscissa, $1/C=1/\text{mole DPN or TPN per liter} \times 10^3$.

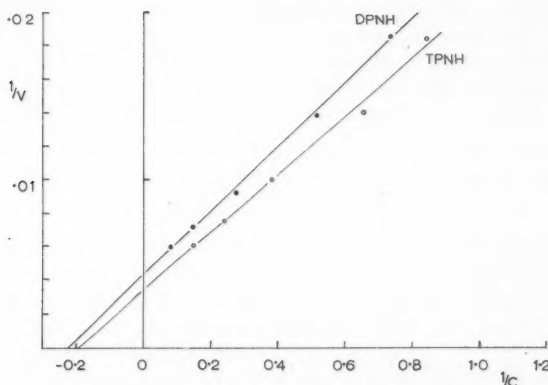


FIG. 4. Rate of the reductive amination of α -ketoglutarate as a function of α -ketoglutarate concentration.

DPN-specific enzyme: $L=1$ cm; $T=22-24^{\circ}\text{C}$; $\lambda=340\text{ m}\mu$. Each cuvette contained 0.23 μmole DPNH, 113 μmoles ammonium sulphate, α -ketoglutarate (concentration in the figure), 0.22 mg purified enzyme and 0.1 *M* Tris, pH 8.3 to 3.0 ml.

TPN-specific enzyme: Conditions same as above. Each cuvette contained 0.2 μmole TPNH, 113 μmoles ammonium sulphate, α -ketoglutarate (concentration in the figure), 0.34 mg purified enzyme and 0.1 *M* Tris, pH 7.5 to 3.0 ml.

Ordinate, $1/V=1/\text{O.D. change} \times 10^3$ per minute; abscissa, $1/C=1/\text{mole } \alpha\text{-ketoglutarate per liter} \times 10^3$.

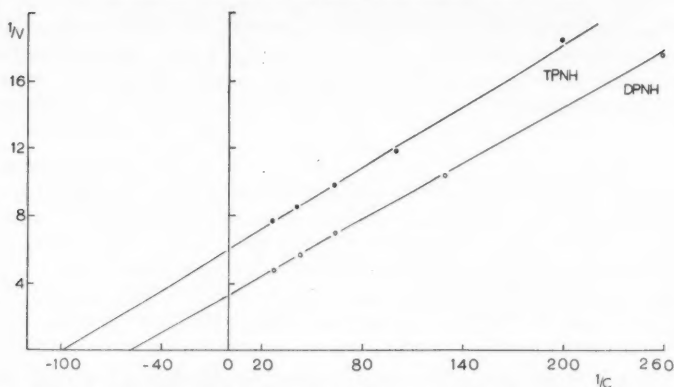


FIG. 5. Rate of the reductive amination of α -ketoglutarate as a function of the ammonium sulphate concentration.

DPN-specific enzyme: $L=1$ cm; $T=22-24^\circ\text{C}$; $\lambda=340$ m μ . Each cuvette contained 20 μ moles α -ketoglutarate, 0.23 μ mole DPNH, ammonium sulphate (concentration in the figure), 0.22 mg purified enzyme, and 0.1 M Tris, pH 8.3 to 3.0 ml.

TPN-specific enzyme: Conditions same as above. Each cuvette contained 20 μ moles α -ketoglutarate, 0.20 μ mole TPNH, ammonium sulphate (concentration in the figure), 0.34 mg purified enzyme and 0.1 M Tris, pH 7.5 to 3.0 ml.

Ordinate, $1/V=1/\text{O.D. change} \times 10^3$ per minute; abscissa, $1/C=1/\text{mole ammonium sulphate per liter}$.

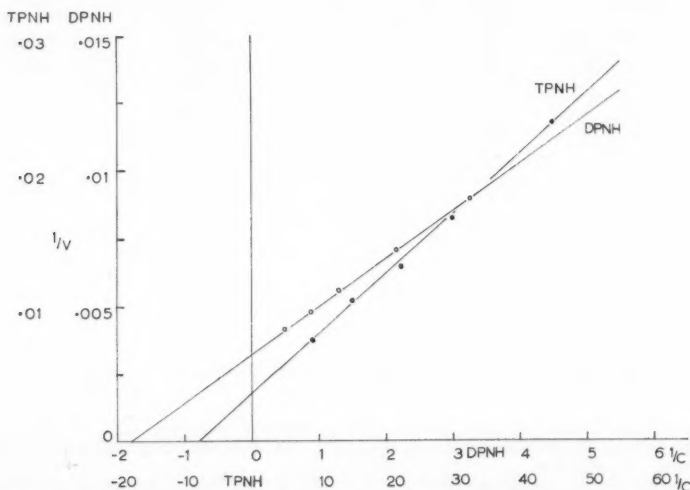


FIG. 6. Rate of the reductive amination of α -ketoglutarate as a function of DPNH or TPNH concentration, respectively.

DPN-specific enzyme: $L=1$ cm; $T=22-24^\circ\text{C}$; $\lambda=340$ m μ . Each cuvette contained 113 μ moles ammonium sulphate, 20 μ moles α -ketoglutarate, DPNH (different concentrations), 0.22 mg purified enzyme, and 0.1 M Tris, pH 8.3 to 3.0 ml.

TPN-specific enzyme: Assay conditions same as above except different concentrations of TPNH (see figure), 0.34 mg purified enzyme and 0.1 M Tris, pH 7.5 to 3.0 ml.

Ordinate, $1/V=1/\text{O.D. change} \times 10^3$ per minute; abscissa, $1/C=1/\text{mole DPNH or TPNH per liter} \times 10^3$.

Michaelis Constants

The dependence of the reaction velocity on the concentration of L-glutamic acid and the oxidized coenzymes in the deamination assay of DPN- and TPN-specific glutamic dehydrogenases is shown in Figs. 2 and 3. The effect of different concentrations of α -ketoglutarate, TPNH and DPNH, and NH_4^+ in the reductive amination assay of both dehydrogenases is shown in Figs. 4-6. The plots have been made according to Lineweaver and Burk (9). In Table III are given the K_m values for the different substrates.

TABLE III
Michaelis constants of TPN- and DPN-specific glutamic dehydrogenases with L-glutamate, α -ketoglutarate, oxidized and reduced coenzymes, and ammonia

Substrate	K_m values in moles/liter	
	TPN-enzyme	DPN-enzyme
L-Glutamate	45×10^{-3}	5.5×10^{-3}
DPN	—	3.3×10^{-4}
TPN	0.5×10^{-4}	—
α -Ketoglutarate	5.3×10^{-3}	4.6×10^{-3}
NH_4^+ ions	10.0×10^{-3}	17.0×10^{-3}
DPNH	—	5.5×10^{-4}
TPNH	1.25×10^{-4}	—

Discussion

In an earlier communication, Sanwal (13) outlined the probable reasons for the presence of two enzymes catalyzing one and the same reaction in certain organisms. Amongst the reasons advanced the more important ones were, first, that the two enzymes, if distributed in the different compartments of the cell could act as a transhydrogenase system (7) as follows.

1. $\text{L-Glutamate} + \text{DPN}^+ \rightleftharpoons \alpha\text{-ketoglutarate} + \text{NH}_4^+ + \text{DPNH} + \text{H}^+$.
2. $\alpha\text{-Ketoglutarate} + \text{TPNH} + \text{NH}_4^+ + \text{H}^+ \rightleftharpoons \text{L-glutamate} + \text{TPN}^+$.
1. + 2. = 3. $\text{TPNH} + \text{DPN}^+ \rightleftharpoons \text{DPNH} + \text{TPN}^+$.

TPN is present in the cells mostly in a reduced form (8) and if these events were occurring in the cell, they would be helpful in liberating oxidized TPN which would then be available for the reactions depending on the presence of oxidized TPN, like the pentose phosphate cycle (8). We do not have any data yet to support this argument.

Secondly, there is a possibility that in the cells one of the enzymes fulfills purely biosynthetic functions and the other enzyme the catabolic ones. In other words, one of the dehydrogenases is exclusively responsible for bringing about the amination of α -ketoglutarate and the other the deamination of glutamic acid. In *Escherichia coli* (14), the L-threonine deaminases, and in *Aerobacter aerogenes* the acetolactate-forming enzymes (6), seem to act in this way. Our unpublished results with some amination-deficient mutants of *Neurospora* point to the second possibility as the more likely one. A comparison of the K_m values of both DPN- and TPN-specific glutamic dehydrogenases for L-glutamate shows that the affinity of this substrate for the DPN-specific

enzyme is indeed very high as compared to the TPN-specific enzyme.

The concept of the organism possessing two enzyme systems, one for biosynthesis and another for breakdown, is quite a recent one (6, 14) for reversible reactions *which are not attended by any large change of free energy*. It is a matter of common knowledge that reaction systems which are accompanied by great changes in free energy require two separate enzymes or enzyme systems, one for biosynthesis and the other for catabolism. The situation presented in this communication, however, is not to be confused with the latter one.

We will present evidence in a later paper that the DPN-specific glutamic dehydrogenase is a catabolic enzyme while the TPN-specific one mostly fulfills biosynthetic functions.

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OBSERVATIONS ON THE MOTILITY AND THE STRUCTURE OF *VITREOSCILLA*^{1, 2}

J. W. F. COSTERTON,³ R. G. E. MURRAY, AND C. F. ROBINOW

Abstract

Two strains of *Vitreoscilla* were studied. Their trichomes showed gliding motility on surfaces and they were immobile in fluid suspension. Motility is the property of the individual cells which make up the trichome, but floating, *living* cells rebounded when they touched a surface. A copious slime layer has been demonstrated and its possible roles in gliding motility is discussed. An association of the unique, scalloped, outer layer of the cell walls of these organisms with their curious motility suggested a new hypothesis: a vibratile, actively undulating surface. Specific antiserum caused rapid cessation of gliding motility; pasteurization and fixation prevented the rebound phenomenon. Stained preparations and electron micrographs of thin sections have provided a general description of structure. This shows, at least, that the classification of *Vitreoscilla* among the bacteria is reasonably founded. Similarities to certain bacteria are seen in the mode of cell division. On the other hand differentiation of central and peripheral cytoplasmic organization is reminiscent of the Myxophyceae, while the diffuse chromatin distribution has precedent in both groups.

Introduction

Colorless, gliding, bacteria-like organisms resembling *Vitreoscilla* were described as early as 1870 by Cohn (7), but they were not named until Pringsheim (26) cultivated and described them. Pringsheim (24) asserted that the gliding organisms of the Beggiatoales, into which the Vitreoscillaceae are placed in Bergey's Manual of Determinative Bacteriology (1), are colorless Myxophyceae. The identification of species within the genus *Vitreoscilla* depends on a key (26) differentiating the species by cellular dimensions, trichome length, rate of motility, and other seemingly variable characteristics.

The *Vitreoscilla* resemble the bacteria in possessing a colorless cytoplasm and in requiring a relatively complex organic medium. They resemble the blue-green algae in having a curious gliding motility, which these organisms share with the desmids, diatoms, blue-green algae, and Myxobacteriales and with other members of the Beggiatoales; this form of locomotion remains an intriguing biological mystery in spite of careful observation (4, 15, 37). And they show similarities to both groups in the size, shape, and arrangement of their cells. Thus they should be helpful to studies of the relationship between these two groups and cytological information is needed.

Materials and Methods

Two strains of *Vitreoscilla* were isolated in 1957 from cow dung. These isolates were obtained by streaking the dung on the agar medium used by

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²The strains used have been deposited in The American Type Culture Collection (numbers 13981 and 13982).

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Pringsheim (25) in his isolation of *Lineola longa* (0.2% sodium acetate, 0.2% yeast extract, 0.2% tryptone) and subsequent picking of the characteristically spreading colonies under the low power of the microscope. Both stock cultures and cultures for experimental purposes were grown on this medium or with vigorous aeration in its liquid equivalent.

Observations of motility were made using a hanging block in a moist, sealed, chamber. Staining of the cytoplasmic membrane was accomplished by the mercuric chloride - Victoria blue and the tannic acid - crystal violet procedures (28 and 30). Lipids were stained with Sudan black B (11) and metachromatic bodies were identified by staining with toluidine blue (14). Chromatin was stained by the HCl-Giemsa (28), HCl - basic fuchsin (35), and HCl-azure-SO₂ (12) techniques as well as by the Feulgen method (23).

Antibodies to these strains of *Vitreoscilla* were induced in rabbits by the injection of cells broken in a Mickle disintegrator (18). These disrupted cells were injected at weekly intervals for 3 weeks and a reinforcing dose was administered 3 months later. The animals were bled by cardiac puncture 2 weeks after this final injection. The presence of antibody was established by agglutination and the ability of the immune sera to make the boundary of the slime layer visible in the phase contrast microscope.

Some preparations for electron microscopy were fixed in 1% osmium tetroxide in distilled water, without adjustment of pH, for 1 hour at room temperature. Others were fixed in 1% osmium tetroxide in *M*/40 tris-hydroxymethyl aminomethane buffer containing 0.4% potassium chloride (20), at a series of pH values between 7.0 and 8.0, for 18 hours at 4° C. Dehydration was carried out in ethanol. The cells were embedded in a mixture of *n*-butyl methacrylate with 10% methyl methacrylate, in which was dissolved 10% by weight of the same material in polymerized form. Polymerization was induced in No. 0000 gelatin capsules by exposing them to a 500-watt ultraviolet lamp (Hanovia) for 5-6 hours at 18 inches, and then holding them at 60° C for 1-2 days.

Other preparations were fixed, embedded in Vestopal "W" (using the optional postfixation treatment with 1% lanthanum nitrate for 1 hour), and dehydrated with acetone according to Kellenberger's (32) standardized techniques.

Sections were cut with a Porter-Blum ultramicrotome, using a glass knife, and examined in a Philips 100-A electron microscope (modified) having 1.8-mm-bore objective pole pieces.

Observations

The *Vitreoscilla* designated as strain I closely resembled the *Vitreoscilla moniliformis* of Pringsheim (26) but the dimensions and shape of the cells of strain II failed to correspond with any of the species described in Pringsheim's key. The cells of *Vitreoscilla* strain I were barrel-shaped (Figs. 1 and 7) and were arranged in trichomes which, in strain I, may attain a length of 960 μ . Cells resembling the akinetes of the Myxophyceae were found regularly in these cultures and cells were seen occasionally having the form of heterocysts. The terminal cells of the trichomes of both strains were morphologically undifferentiated.

Nutrition

If acetate was omitted from the medium both strains of *Vitreoscilla* failed to grow. Acetate could not be replaced by propionate, lactate, ethanol, citrate, or succinate. And the addition of dextrose, galactose, xylose, dulcitol, glycine, cystine, or glutamate failed to support growth in the absence of acetate. When acetate was included in Pringsheim's medium, growth was slightly stimulated by the addition of glycine or of alanine but not by the addition of cystine. The organisms grew poorly on potato agar and failed to liquefy gelatin, reduce nitrate, generate hydrogen sulphide, or change litmus milk.

Motility

The motility of the *Vitreoscilla* on agar was consistent with classical gliding motility and was best seen in strain I. A 10-cell trichome was observed to glide steadily at a rate of 23μ /minute for 15 minutes before the trichome halted and reversed its direction. Trichomes did not distort markedly until an obstruction was encountered. In some cases they reversed their direction of movement; in other cases bending allowed circumvention of the obstacle. This bending of the trichome was accomplished by flexion at the intercellular junctions and not by a distortion of the cells themselves.

In addition to their gliding motility, which is dependent on the availability of a surface, these organisms carried out a rolling and darting jerky movement in *liquid* cultures. This "motility" bears little resemblance to that caused by flagella. It may be of some importance in understanding gliding motility because the jerks seemed to occur when the cell touched a surface such as the slide or the cover slip. It was seen to be an attribute of living cells because both pasteurized and chemically fixed suspensions did not show the phenomenon.

The lengths of the cells of these trichomes were measured during active gliding motility and no elongation or contraction of the cells could be detected. The behavior of India ink particles in contact with moving trichomes did not suggest the movements that would be expected if gliding motility were due to the extrusion of slime or to the release of a surface tension reducing substance. There were no localized regions of turbulence such as occur as a result of flagellar action. Gray's flagella stain and electron microscopy of whole cells failed to reveal the presence of flagella, cirri, or structures suggestive of an organ of locomotion.

The behavior of the trichomes in negotiating the tortuous passages between carborundum particles deposited on the medium was informative because it showed that the mechanism of motility was not confined to one area of the trichome. If the leading end of the trichome became immobilized, the trailing end continued to move until the trichome was markedly bent or "kinked". When the leading end was freed, the "kink" was gradually straightened out and the trichome continued to move in a manner indicating that the leading portion also has the ability to glide. Gliding motility cannot, therefore, be considered the function of a special terminal cell or set of cells either pushing or pulling the whole trichome.

Long-continued observations showed that when trichomes moved into a new area their direction was random and their rate of motility was relatively

slow. However, when other trichomes encountered the invisible "track" of their predecessors at a suitably oblique angle, they changed their direction to move along this "track" at an increased speed and did not leave it unless forced to do so by another trichome. In fact when "tracks" were well established on the agar, and trichomes met on these well-travelled trails, cells were easily exchanged from one trichome to another, and leading units of as many as five cells were seen to pull away from their trichomes due to a greater rate of motility. Trichomes composed of two cells were seen to move very slowly. Thus it was concluded that the trichomes of *Vitreoscilla* are composed of individually gliding cells whose attachment to their neighboring cells is weak. This rather resembles the general behavior of motile colonies of *Bacillus* species (21).

Elasticotaxis (34) was not demonstrable using these strains of *Vitreoscilla*.

The slime layer of these organisms was delineated only poorly by the use of Dyar's anionic detergent-staining method, but elegant preparations were obtained with immune serum by Tomcsik's method (35). The slime layer was easily seen by phase microscopy after exposure of the trichomes to a specific immune serum (Figs. 1 and 2). The specificity of this reaction was such that antibodies induced using strain I cells would not react with the cells of strain II and vice versa.

The gliding motility of the trichomes stopped abruptly upon exposure to the specific immune serum, while non-immune serum slowed motility gradually until its cessation after approximately two hours. Specific antibodies caused lysis of the cells of each strain within 2 hours while non-specific and non-immune sera caused lysis at a much slower rate. Figure 2 shows the large amount of slime found in densely colonized areas while Fig. 3 shows an isolated trichome with less accumulated slime.

The slime layer failed to react with potassium permanganate, iodine, alcian blue (38), ninhydrin-Schiff (38), periodic acid-Schiff (38), Sudan black B, and bromphenol blue (17) before or after mild hydrolysis using H_2SO_4 , HCl, or periodic acid. It was not dissolved by absolute alcohol or ether.

Structure

Cell Wall

Pringsheim has based his statement (26) that the *Vitreoscillaceae* do not have a rigid cell wall on observations of flexion of the cells of some species during their gliding motility, the weak refractility of unstained cells, and his inability to see the cell wall when these organisms were plasmolyzed.

Electron microscopy of thin sections revealed a cell wall similar to, and no more substantial than, those of other Gram-negative bacteria. The cell walls of *Vitreoscilla* are made up of an inner, dense layer (~ 30 Å) separated by a relatively wide zone of low scattering power from a fine and distinctly folded outer layer of about 50 Å thickness (Figs. 8, 10, 15). The total thickness of the cell wall varied between 150–400 Å because of the folding of its outer layer. Figure 9 shows this structure at very high magnification. The less electron-dense central space of the cell walls was filled with a substance more dense than the embedding plastic, and no "empty" spaces were seen in this area, which may perhaps be called a layer in spite of its irregularities of thickness. The

"scalloped" outer layer was seen in all of five different embeddings fixed at different pH values, in cells which showed no evidence of shrinkage beyond that degree inevitably seen in methacrylate-embedded cells. An identical folding of the outer layer of the cell walls was seen in cells fixed by Kellenberger's method and embedded in Vestopal (Fig. 11). Measurement showed that very little shrinkage had occurred, and so the creniated outer layer was considered to be real rather than artifact. The folding of the outer layer is seen in both longitudinal sections and cross sections, which suggests that it is not preferentially oriented relative to any one axis of the cell. Examination of shadowed cell walls prepared by breaking the cells by means of a Mickle disintegrator failed to reveal any recognizable undulations in their outer layer (Fig. 6), and attempts at carbon replication were similarly non-informative about the surface.

Plasmolysis (Fig. 4) was accomplished by exposure of the cells to a saturated solution of sodium chloride for 10 minutes, followed by staining with Lugol's iodine saturated with salt. Cells that had been air dried and subsequently stained with neutral red showed retraction of the cytoplasm away from a rigid cell wall. Figure 5 shows an empty cell wall, which clearly demonstrates the rigidity of this structure.

Cell Division

Both living and stained (Fig. 7) preparations suggested that the cells of *Vitreoscilla* divide by a process of constriction rather than by septum formation. This was substantiated by electron microscopy. Sections of cells of both strains of *Vitreoscilla* fixed during division show various stages of an annular invagination of the inner dense layer of the cell wall, followed more or less closely by the outer dense layer (Figs. 8, 11, 14, and 15).

Cytoplasmic Membrane

The cytoplasmic membrane of the *Vitreoscilla* was not different from the cytoplasmic membrane of the Eubacteriales when stained by the Victoria blue and tannic acid-crystal violet techniques (30). It was easily demonstrable in plasmolyzed cells stained with iodine (Fig. 4) where it was recognized as a broad contour at the surface of the contracted protoplast. Plasmolysis causes the protoplast to retract, in a number of places, to form bay-like indentations into the cytoplasm, and the cell wall can just be seen as a thin straight line where it bridges these concave areas of retraction. Some electron micrographs of methacrylate-embedded cells (Figs. 8, 9, and 10) showed a very fine, membrane-like structure under the inner dense layer of the cell wall. A layer of low scattering power separated it from the inner table of the cell wall. However, this structure was not identified in Vestopal embeddings.

Cytoplasm

The cytoplasm of *Vitreoscilla* consists in large part of closely packed spheres and rodlets very similar to those seen in thin sections of a variety of other bacteria (18, 3). The packing of these components appears to be slightly closer and more regular in the region immediately under the cytoplasmic membrane (Figs. 8, 10, 14, and 15) and this area, which is approximately 0.1μ in width, contains fewer of the very electron-dense granules scattered throughout the rest of the cytoplasm. In occasional micrographs the components of this area

of the cytoplasm appear to be arranged in a fine palisade parallel to the surface and unrelated to the direction of section cutting. But, considering the possibilities of fixation artifact and the "pearl string" effect, the existence of a specific organization at this level was discounted.

Fixed cells from young cultures are uniformly stained by Giemsa solution unless they are first treated with 1 *N* HCl. Ageing is accompanied by the gradual loss of basophilic matter from the periphery of the cells and a corresponding concentration of the electron-dense granules in inner regions of the cell is seen in electron micrographs of sections (Figs. 12 and 13). The peripheral areas from which these granules have moved show a very uniform fine structure and a relatively weak electron-scattering power. This material may well be the "ground substance" of the cytoplasm of these cells.

Experiments on the effects of changes in the ionic environment of the cells (39) showed that manipulation of this factor alone did not produce centralization of the basophilic area. Vital staining on 24-hour cultures with neutral red did not show distinct differentiation of the central area of the cytoplasm.

Metachromatic bodies were regularly seen in stained preparations and they appeared to coincide in arrangement with electron-dense bodies, which tended to volatilize in the electron beam (31). Non-granular areas, similar in form to the chondrioid shown by Kellenberger in *Escherichia coli* (13), were found in some sections of cells fixed by Kellenberger's method and embedded in Vestopal (Figs. 11, 17). However, lamellated cytoplasmic structures of the types shown in *Mycobacterium* (33), *Streptomyces* (10), *Bacillus* (9), and a variety of other bacteria were not seen in either methacrylate or polyester embeddings, nor have we seen any simpler intrusions of the cytoplasmic membrane.

Staining with Sudan black B showed occasional lipid inclusions. These were identifiable in Vestopal embeddings as rounded cytoplasmic spaces having a dense margin (Figs. 11, 17).

Chromatin

The chromatin of both strains of *Vitreoscilla* was stained by the HCl-Giemsa, HCl-basic fuchsin, and HCl-azure-SO₂ methods in preparations fixed at intervals from 1 hour to 10 days after subculture on fresh medium. In all cases the chromatin was in the form of fine particles that were evenly distributed throughout the cytoplasm. The Feulgen test was only faintly positive, probably because of the smallness of the particles and their dispersion in the cell. The most extreme examples of this even distribution of chromatin was seen in the oldest cultures and in the akinete-like structures.

However, a Feulgen-positive central aggregate of chromatin was easily seen in cells that had been exposed to 10% sodium chloride in water for 10 minutes. The chromatin of salt-treated cells was also stainable by the HCl-Giemsa and HCl-azure-SO₂ techniques, and was seen in electron micrographs as a large, central, electron-translucent area containing coarse, electron-dense structures. So the seeming lack of chromatin in cells in the earlier experiments was illusory.

Electron-translucent zones enclosing relatively coarse, electron-dense, filaments were seen in sections from methacrylate embeddings and had a distribution (Figs. 8, 10, 14, 15) that paralleled that of the particles of chromatin seen in stained preparations. In some sections the electron-dense bodies, which

were regularly seen in these cells, appeared to be associated with the chromatinic areas and with the coarse, electron-dense, filaments in particular. The filaments seen in the chromatin areas are much finer and more evenly distributed (Figs. 11, 17) in cells fixed by Kellenberger's method and embedded in Vestopal, but the chromatin areas themselves show the same order of dispersion that was seen in methacrylate preparations.

Discussion

Observations of motile trichomes of *Vitreoscilla* have led to the conclusion that the cells making up these trichomes are individually motile, and that the trichome is neither pushed nor pulled by the action of a specialized terminal cell or group of cells. It is tempting to attribute an active role in gliding motility to some superficial structure because of the abrupt cessation of motility caused by the exposure of the trichomes to specific antibodies.

The slime layer may contribute to "track" formation by the trichomes and to the ability of seemingly unconnected cells to move as a unit. However, the extrusion of slime (15) seems inadequate to account for a rapid rate of gliding with occasional reversal of direction. Alternatively, the slime layer may function as a lubricant to aid in overcoming the forces of surface tension and friction, or it may act as a necessary special menstruum in which the as yet undiscovered organ of locomotion must function. The explanation of gliding motility as a product of the anisotropic swelling of slime (8) was strongly contraindicated when Niklitschek (22) showed that the slime layer of *Oscillatoria* did not behave anisotropically.

The peculiar folding of the dense outer layer of the cell wall interested us because of the absence of any similar appearance in the walls of non-gliding, Gram-negative, bacteria. This suggested a different and, we believe, a new mechanism of gliding in the seeming absence of flagella or of cirri. The hypothesis is that the motility of these organisms is a function of orderly waves of contraction in an elastic outer layer of their cell walls. A rhythmic microscale undulation of this superficial layer could account for the gliding motility of *Vitreoscilla* in contact with a surface, for an apparent inability to swim in fluids, and for the rebound phenomenon when a solid surface is touched.

Direct experimental support is lacking. The main merit of this hypothesis is as an alternative to a list of previously considered possibilities.

The cell walls of all the gliding organisms we have studied (the Myxophyceae, *Myxococcus xanthus*, *Leucothrix*, and *Beggiatoa*) have shown a crenated outer layer. But undulations of the same order of magnitude are also seen in the wall of *E. coli* fixed according to Ryter and Kellenberger (32a). The cell wall of *Vitreoscilla* resembles those of many other Gram-negative bacteria in having a complex ultrastructure, rigidity, and poor staining properties. And a further similarity is the accomplishment of cell division by a process of constriction. In *Escherichia coli* the three layers of the cell wall constrict as a unit to effect cell division (28, 13). However, in *Vitreoscilla*, the constriction of the outer dense table of the cell wall is not necessarily perfectly synchronized with that of the inner layer and is often quite incomplete when the inner layers of the cell walls of the daughter cells are fully formed. The mechanism of these constrictions cannot yet be deduced from the

evidence at hand, and there was no sign in any embedding of "peripheral bodies" (6) or the lamellated organelles thought to accompany septum formation (9).

The cytoplasm of *Vitreoscilla* is seen to have the same order of structure as that observed in a variety of the bacteria. It is remarkably free of membranous intrusions and membranous structures, resembling the enteric bacteria in this and the possession of a peculiar chondrioid (18, 12). Of the greatest interest was the observation that the cytoplasm contains a peripheral segment of recognizably different behavior and structure. This will require further examination at the highest resolution but, leaving aside the more dubious possibility of parallel arrays, this sort of differentiation reminds one of the protoplast of blue-green algae where there is a centropasm (containing both RNA and DNA) and a peripheral chromatoplasm. Pringsheim (24) may then be correct in looking upon these organisms as colorless blue-green algae that have acquired a nutritional habit more akin to that of the bacteria. This fascinating question remains open to more certain proof.

Vitreoscilla is one of the bacteria that have been called "awkward" because no discrete chromatin bodies are formed at any stage in the life of the organism (29). The dispersed and particulate chromatin of these cells is identical with that of the bacteria with regard to staining properties and appearance in electron micrographs of thin sections (27, 2). And the chromatin of these strains of *Vitreoscilla* reacts to changes in ionic environment in the same manner as in the bacteria. If the whole cytoplasm of bacteria can be equated to the whole centropasm of blue-green algae, then the chromatin distribution in the latter, so elegantly described by Cassel and Hutchison (5) and others, must also be considered to be diffuse. Neither staining nor electron microscopy of thin sections has yet revealed any change in the nature or distribution of the chromatin particles which might precede or accompany cell division. It can be reiterated, as in the case of the Myxophyceae and the "awkward" bacteria (29), that the mode of division of their chromatin is not easily imagined.

Thus the Vitreoscillaceae provide a most interesting taxonomic isthmus. Their resemblance to the Myxophyceae includes their gliding motility and certain aspects of their cytoplasmic organization, whereas their lack of photosynthetic pigments, the ultrastructure of their cell walls, their mode of cell division, and their nutrition suggests some relationship with certain of the bacteria. These organisms resemble both some of the Myxophyceae and certain bacteria in the size and shape of their cells, as well as showing a distribution of chromatin similar to that of certain members of both of these two large groups of organisms. It is our belief, however, that the case of the *Vitreoscilla* is most important in drawing attention to the peculiar similarities, including the finest of structure, between bacteria and blue-green algae. We must hope for more revealing studies of all these organisms.

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EXPLANATION OF FIGURES

NOTE: All figures show *Vitreoscilla*, strain I. Strain II was not different in the features shown here, except for smaller cellular dimensions. Markers indicate 5 μ on photomicrographs and 1 μ on electron micrographs unless otherwise indicated.

FIG. 1. Living trichomes photographed in dark-phase contrast 5 minutes after exposure to a specific immune serum. This appearance differs from that of untreated cells only in the distinctness of the enveloping slime layer.

FIGS. 2 and 3. As in Fig. 1 except that the cells have been exposed to immune serum for 15 and for 60 minutes, respectively. Note the "clearing" effect on the cytoplasm, which correlates with impaired viability; ultimately there is lysis.

FIG. 4. Cells plasmolyzed in Lugol's iodine saturated with sodium chloride. The cytoplasmic boundary is clearly visible; the cell wall is barely seen.

FIG. 5. Cells from a 24-hour culture stained with neutral red. Note the rigidity of the empty cell wall (arrow). The peripheral zone may be an optical artifact due to the cell wall and slime layer.

FIG. 6. Micrograph of an isolated cell wall shadowed with tungsten oxide. The cell was disrupted by shaking with "Ballotini" beads in a Mickle apparatus. No regular folding of the cell wall is visible.

FIG. 7. Cells stained with Victoria blue, after treatment with mercuric chloride, to show the cell surface (cytoplasmic membrane and cell wall). The figure shows progressive stages of constrictive cell division (e.g. cells a-d) and also large akinete-like cells.

FIG. 8. Micrograph of a section (methacrylate embedded) illustrating cell wall structure, cytoplasmic membrane, and the disposition of protoplasmic components. The cell wall shows a crenated outer border and at least three components. The cytoplasmic membrane has a low density component (arrow) causing an apparent spacing of the dense component and the inner cell wall. All the membranes take part in the constrictive cell division (see also Figs. 11 and 15), but the angle of cut determines what is seen of them. The protoplasm contains a number of small volutin granules (M), as well as condensed elements of the chromatin body (n) and ribosomes throughout the cytoplasm.

FIG. 9. An enlargement of a portion of Fig. 8. Some cytoplasmic elements are visible and the dense granules have the size and form of ribosomes.

FIG. 10. Micrograph of a section of a newly divided pair of cells showing structures similar to those in Fig. 8. A narrow zone (arrow) in the periphery of the cytoplasm seems to have a less dense granularity than the rest.

FIG. 11. Micrograph of a section of a dividing cell fixed and embedded (Vestopal) after Kellenberger. The dense, non-granular chondrioid (Ch) and lipid inclusions (L) are obvious, as is the reticulated chromatin area (n). See also Fig. 17 and compare with Figs. 8, 10, 15, and 16.

FIG. 12. Micrograph from a methacrylate embedding of a 3-day culture. It should be noted that the chromatin body (n) is less disperse and the contents extremely condensed. The absence of the dense, granular, cytoplasmic components from the periphery of the protoplasm is very marked (arrow).

FIG. 13. Similar to Fig. 12 but fixed and embedded according to Kellenberger. The chromatin body (n) is filled with a reticulum (n). The peripheral zone (arrow) is visible but shows a less distinct boundary than shown in Fig. 12.

FIG. 14. A portion of a methacrylate section to show the seeming differentiation and linear arrangement of particles (arrow) in the periphery of the cytoplasm.

FIG. 15. Section (methacrylate) of a partially divided cell showing the pattern of constriction. It also shows a slightly differentiated peripheral cytoplasmic zone.

FIG. 16. An akinete-like cell in a section (methacrylate), to show the wide dispersion of the chromatin elements (e.g. n). From the way these behave in salt solutions it is con-

cluded that the chromatin forms a continuous meshwork in the cytoplasm. There are several volutin granules (m).

FIG. 17. A section from a Vestopal embedding, fixed according to Kellenberger. Lipid inclusions (L) and a chondrioid (Ch) are shown. The chromatin material (n) can be identified in a number of areas of the cell once its form is recognized, and it seems to be fully as disperse as in the methacrylate sections.

NOTE: Figs. 1-17 follow.

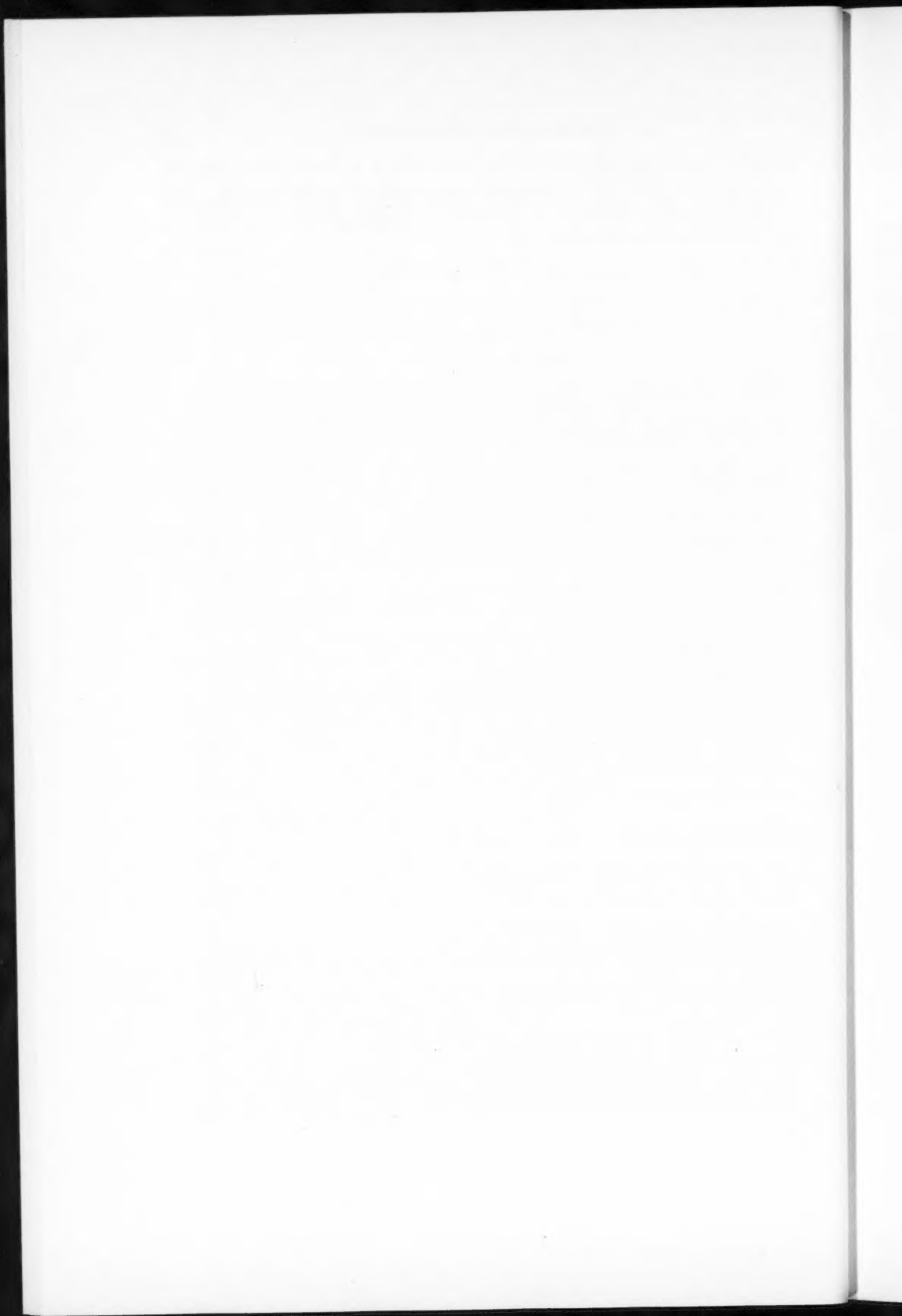
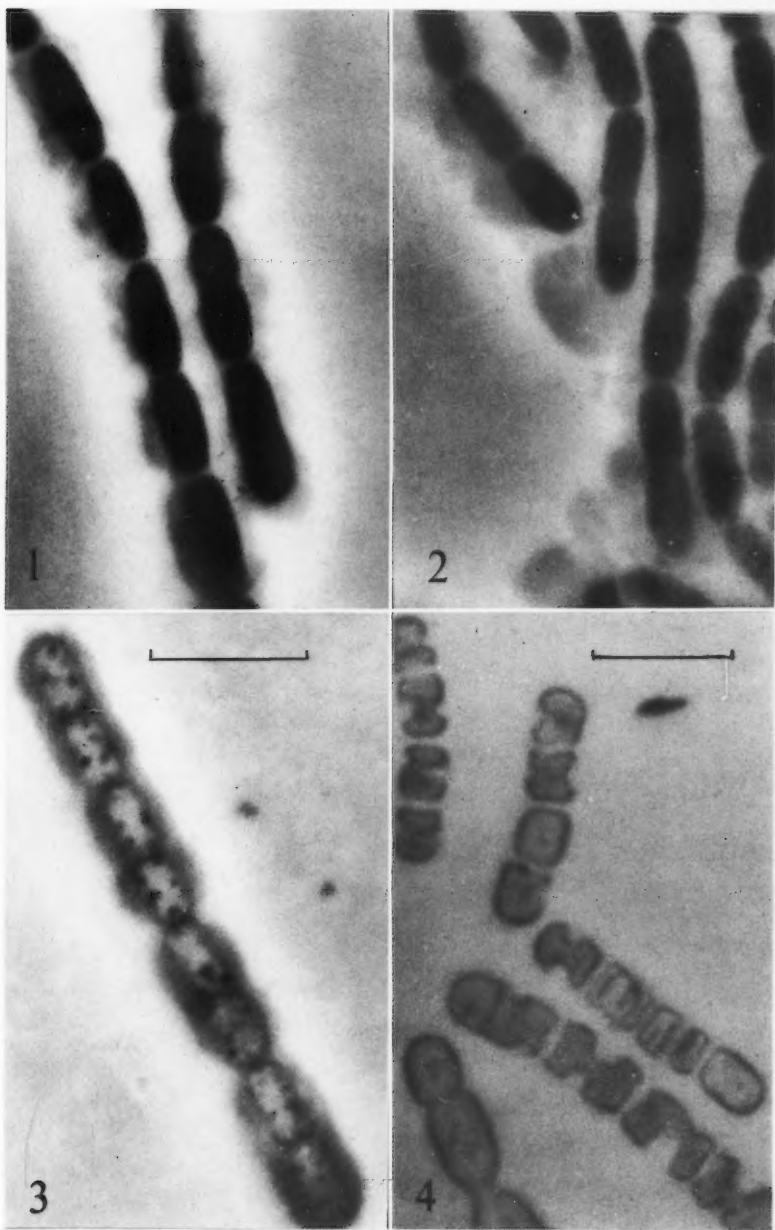
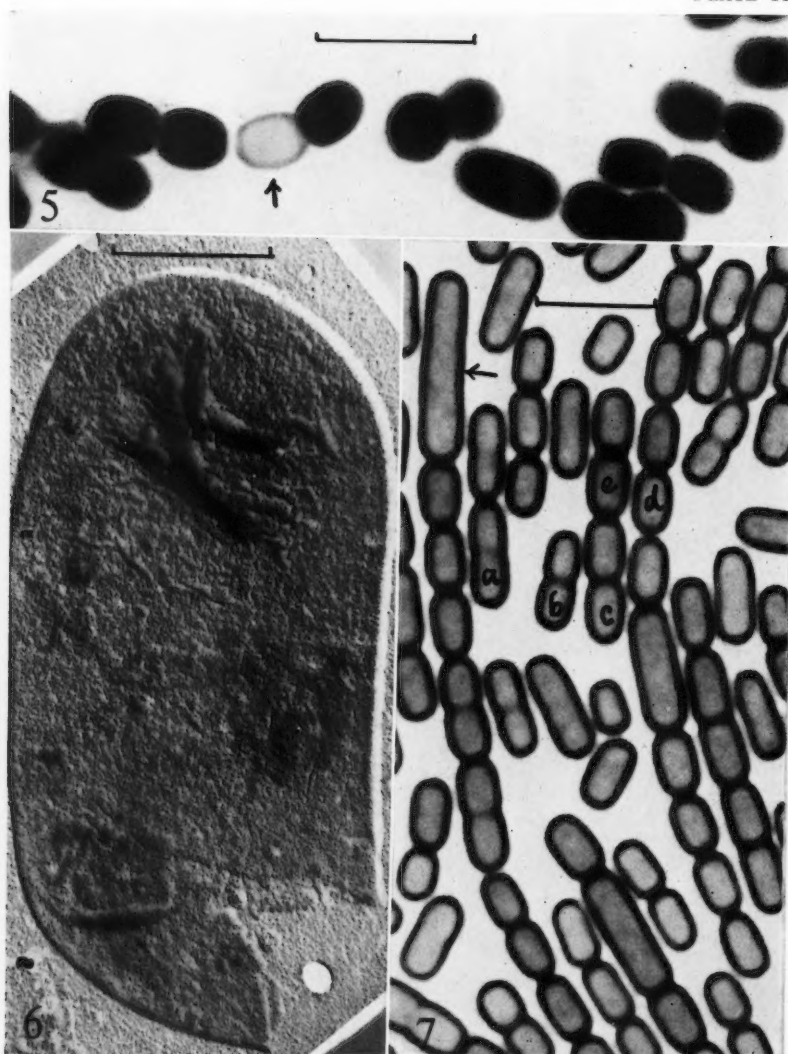
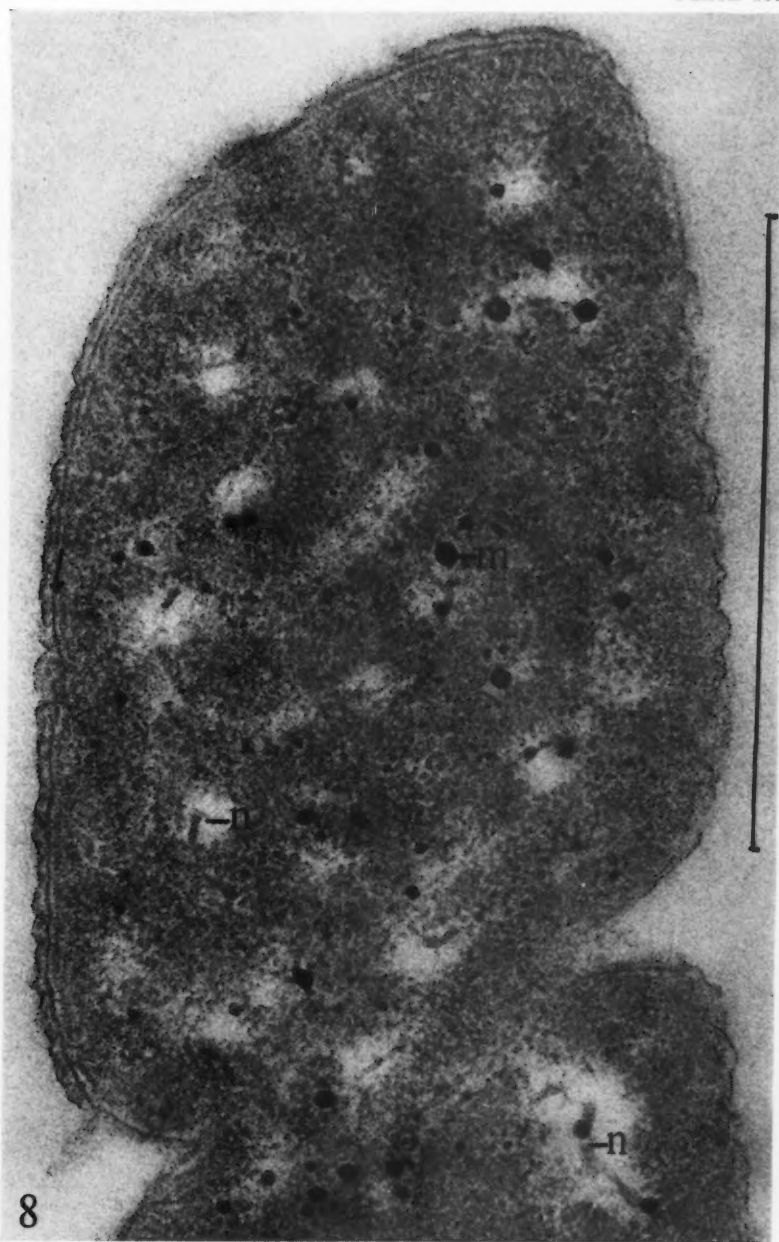


PLATE I





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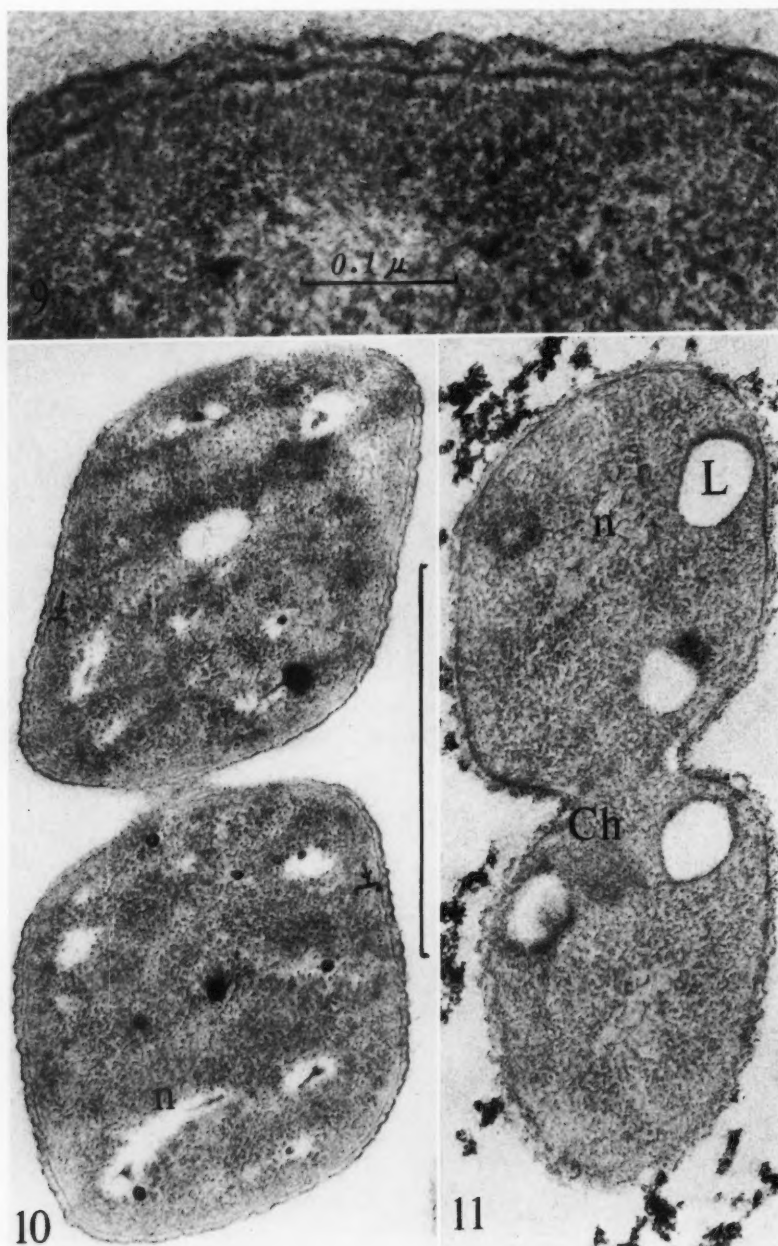
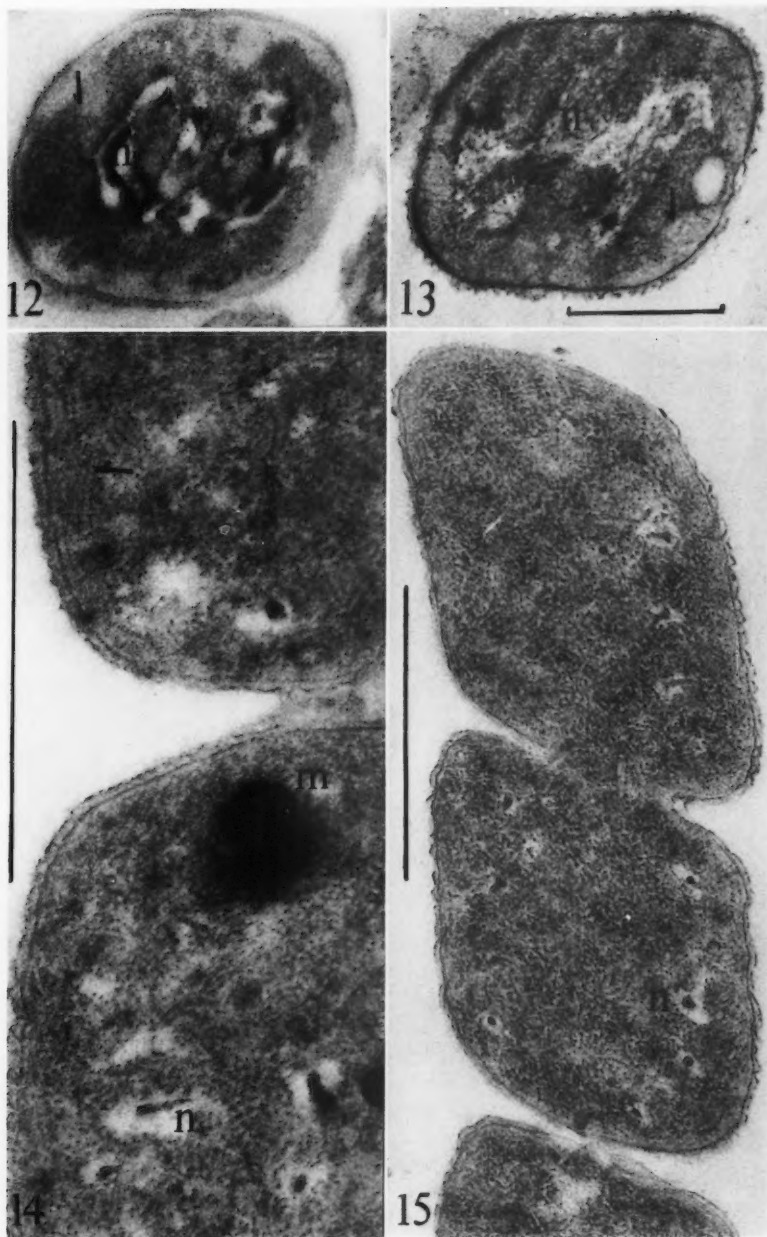
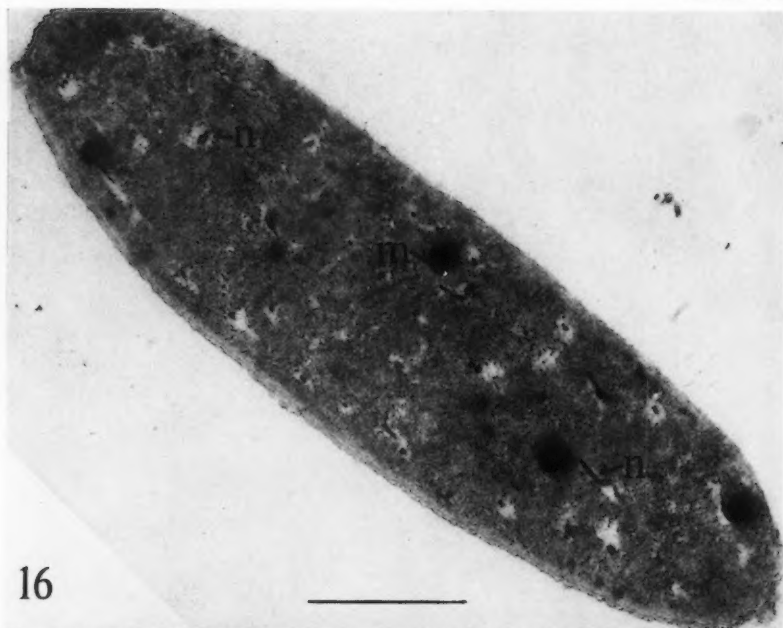
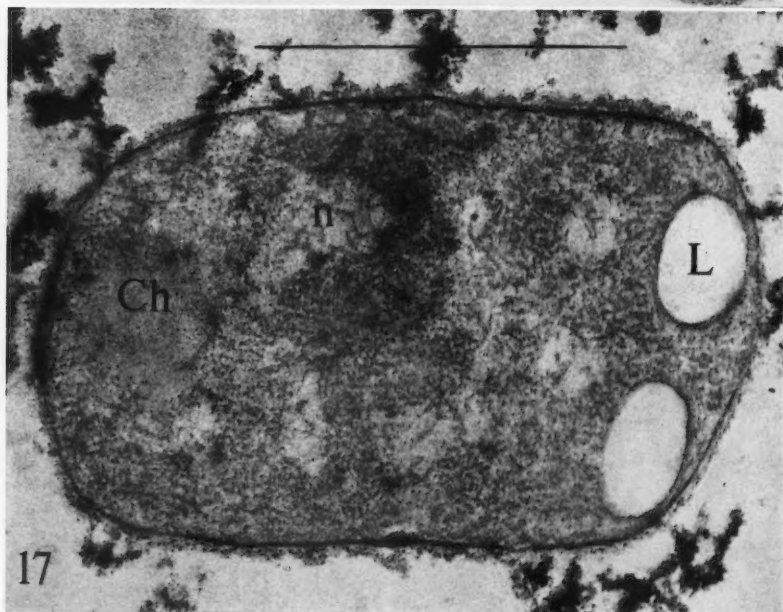


PLATE V





16



17

FERMENTATION OF SULPHITE WASTE LIQUOR TO PRODUCE ORGANIC ACIDS¹

M. E. MARTIN, M. WAYMAN,² AND G. GRAF³

Abstract

Continuous fermentation of sulphite waste liquor by *Propionibacterium arabinosum* was accomplished in a system with bacterial population maintained in suspension (3.0×10^8 cells per ml) in circulating liquor, and partially as visible colonies on lump limestone. The limestone served to maintain optimum pH in the fermenting waste liquor. When the population was established, no growth substances were necessary in waste liquor feed. Recycling a portion of the fermented liquor was beneficial in obtaining rapid adjustment of pH through reaction of calcium salts of organic acids with acidic components of fresh waste liquor. The calcium lactate present in recycle liquid stimulated rate of conversion of fermentable sugar to volatile acids and aided in pH control. Conversion of fermentable sugars to volatile acids was obtained at 83.0 to 86.5% efficiency in 55 hours retention time. The volatile acids were propionic and acetic acids in 2:1 mole ratio.

Introduction

A continuing interest in possibilities for utilizing fermentable sugars from sulphite waste liquor (2, 5) has existed for some time. Commercial activities of recent years focused attention on propionic acid, a raw material for producing cellulose propionate used in manufactured plastics of excellent quality, and salts of propionic acid widely adopted as mold inhibitors. These developments were responsible for the present investigation.

Beginning with the pioneer work of Orla Jensen (4) in 1898, all the investigators comment on the slow rate of fermentation with *Propionibacterium*. In his comprehensive monograph, Van Niel (10) suggested that the application of propionic acid bacteria for the production of propionic acid might be possible. The difficulty encountered in producing propionic acid by fermentation, apart from slow growth of the microorganism, is maintenance of a high population level to support conversion of fermentable sugars to organic acid. For rapid fermentation, adequate control of pH and temperature must be achieved.

Van Niel took advantage of the sedimentation of propionibacteria by letting the raw material flow slowly through a bottom layer of microbes. In developing a rapid, continuous process, all the factors affecting this type of fermentation were taken into account to provide an automatic system.

The process was based on: (1) a long path of raw material through tubes filled with limestone lumps on which the inoculum was precipitated, (2) a heavy initial inoculum grown on nutrients in separate containers, and (3) a partial recycling of the fermented waste liquor.

Materials and Methods

Raw material for the fermentation was waste liquor for sulphite wood-pulp production. During the course of these trials waste liquor containing from

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1.8 to 2.5% fermentable sugars were used. The Schoorl-Menzinsky (6) method was used for measuring sugar content of sulphite waste liquor.

A major difficulty with sulphite waste liquor is its acid content (sulphurous acid and lignin sulphonic acid). Boiling the liquor for 50–60 minutes under slight aeration stripped the material of free sulphurous acid, which is the major microbial poison. This operation raised the pH from 1.3–1.5 to 2.5–3.0. The residual acid was neutralized on the limestone surfaces (8) in the first segment of the fermentor system. Recycle of a portion of the stream leaving the system and blending this liquor, carrying calcium salts of organic acids, assisted in rapidly increasing the pH of the incoming stream of waste liquor as it entered the continuous fermentor. Except for the initial charge carrying the inoculum into the fermentor, no further treatment was necessary. In the first charging operation, the liquor was neutralized (pH 6.8–7.0) by adding CaCO_3 to the stripped waste liquor.

The inoculum was prepared from a culture of *Propionibacterium arabinosum* (American Type Culture Collection No. 4965) (11). An agar stab culture was transferred first to a liquid medium (2% glucose, 2% yeast extract, and 2% calcium carbonate precipitate in sterile water). After an initial incubation during an interval of 2–3 weeks, it was transferred serially several times in media of the same composition. In the final step this glucose culture was inoculated to a sulphite waste liquor basal medium containing 1.8% sugar, 1% ether extract of yeast extract, 0.5% dibasic ammonium phosphate, 0.5% urea, 1.5% calcium carbonate precipitate, and 10 p.p.m. each of cobalt, manganese, magnesium, and iron ions respectively. Within a week the population attained a concentration of $3.0\text{--}3.5 \times 10^8$ cells per milliliter. The same level was kept and even surpassed during fermentation. The bacterial concentration was determined by direct microscopic count using a Levy counting chamber No. 500, which, although designed for yeast, served very well for counting bacterial cells. Counts did not vary more than $\pm 5\%$ between replicate samples.

Growth of *P. arabinosum*, in the presence of trace elements, was promoted by the use of yeast extract, by its ether extract, or by various nitrogen sources (9, 12). These supplements were used in the medium to grow 2 liters of culture as a vigorous inoculum for addition to the 5-liter storage vessel. The continuous fermentation started when a slow flow rate from this reservoir entered the multitube system. The fermentor, Fig. 1, consisted of five vertical glass tubes, 1 in. \times 6 in., connected in series. Tubes 1 to 4 were packed with limestone granules of approximately 1/4 in. mesh size. Analysis of the limestone showed 43–44% loss on ignition, 99.4–99.7% calcium carbonate. Small amounts of impurities such as silica, aluminum, magnesium, manganese, and iron compounds were present in the limestone. The last tube in the system, Fig. 1, contained no limestone, but served as a sedimentation unit to trap any particulate material carried along with the stream. Upward flow was maintained in the system by connecting the overflow from each unit to the bottom inlet of the tube which followed. The limestone-packed tubes were surrounded by water jackets supplied with circulating water thermostatically controlled, $35^\circ\text{C} \pm 1$. Each unit in the multitube system was provided with a thermometer, and top gas-relief line. The latter reached high enough to be in hydrostatic equilibrium with storage tank level. Sterile cotton plugs were used in

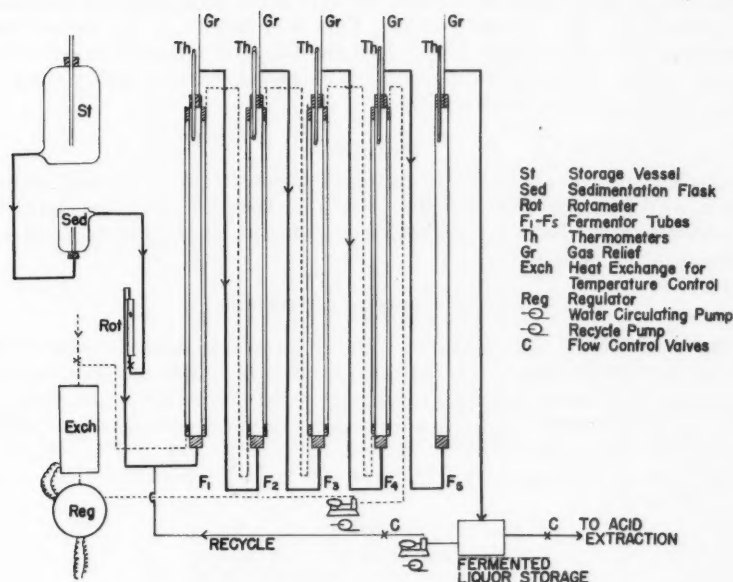


FIG. 1. Laboratory scale continuous fermentation unit.

vent and vacuum-break tubes to prevent atmospheric contamination.

After liquid culture was added to the system, it was completely filled by charging the storage vessel with 3.5 liters of previously neutralized, pH 6.8-7.0, sulphite waste liquor which was slowly streamed into the unit through a very small rotameter. Then the regular operating pattern was started by adding daily sufficient air-stripped, 3.5 pH, sulphite waste liquor to restore the level to the 3.5 liter mark in the storage vessel. Daily output was slowly increased to 2 liters and steadily maintained at this level of operation with 50%, 1 liter, of the output recycled to storage, and the remainder, 1 liter, was withdrawn for analysis. Continuous operation was maintained for sufficient time to reach equilibrium conditions. During the trial, surfaces of the limestone were covered with characteristic yellow-brownish spots which were found to be vigorous colonies of *P. arabinosum*. The standard rate of operation gave a retention time of 54-55 hours.

Analyses were conducted on the continuous fermentor output to determine yield and degree of conversion. The crude acid (in the form of its calcium salts) is of complex nature. Total acid was measured by titration after ether extraction from a sample of fermented waste liquor previously reacted with mineral acid. Ether extraction was made by processing 78 hours continuously in a liquid-liquid extractor. Determination of volatile acids was done on steam-distilled material from another portion of fermented waste liquor sample previously reacted with mineral acid.

There was qualitative evidence that no other acids than propionic and acetic were present in the volatile portion. This was confirmed by vapor-phase chromatography. It was found useful to use Duclaux numbers based on the different steam volatility characteristics of propionic and acetic acids to determine their ratio in control testing.

Results

According to theory, the fermentation of sugars to propionic acid yields 2 moles of acid for each mole of sugar (3, 7, 13). Therefore S millimoles of fermentable sugar produces $2S=A$ millimoles of acid. If A' millimoles of acid are found in the product:

$$A'/A = \text{degree of conversion.}$$

The grams of volatile acids found in 1 liter of fermented waste liquor if multiplied by 10 and divided by the percentage of fermentable sugars present in the original sample provides the percentage yield of volatile acids. Since the molar ratio of propionic to acetic acid was 2:1 and independent of the degree of sugar conversion, see Table I, an average mole weight of 70 (HPr = 74 and HAC = 60) was used. On this basis the highest theoretical yield of volatile acids was 77%.

TABLE I
Ratio propionic to acetic acid at varying
percentage conversion of fermentable sugars
to volatile acids

Degree of conversion	HPr:HAC
55	2.05
71	1.90
75	2.15
85	2.00
87	1.98

Table II presents a record of a continuous operation trial. No recycling of fermented liquor was done during the first day. The bulk of the output was recycled through the system until the fifth day. Then the operation was held steady with 50% recycle of fermented waste liquor. This decreased the retention time to 54-55 hours while maintaining a conversion rate of 83-85%. Factors responsible for these results include control of temperature, pH, and maintenance of an abundant bacterial population. It was found that recycling, as practiced, was an important contributing factor in achieving the required conditions.

The low-velocity flow of waste liquor through the limestone packed tubes provided a steady interaction between bacteria and substrate, under controlled pH and temperature conditions. These optimum conditions also prevented an unfavorable aerobic type dissimilation. The limestone surfaces were well covered with colonies of *Propionibacterium*, resulting in a high probability of contact. Adsorption of substrate molecules may also facilitate a "catalytic" increase in the speed of conversion. Neutralization of the acids produced takes place instantaneously on the limestone surfaces, without any local areas of

TABLE II
Operating scheme of continuous propionic acid fermentation of sulphite waste liquor by *Propionibacterium arabinosum*

Days of operation	Daily output in ml	Recycled, ml	pH of fresh liquor*	Original sugar, mmol/l.	Sugar left, mmol/l.	Acids, mmol/l. volatile	Degree of conversion, %	Yield, %†	Retention time, hr
Days of operation									
1	800	None	6.8-7.0	100	10.5	150.0	75.0	58.0	65-70
2	1100	1000	2.5-3.0	100	11.1	170.0	85.0	66.0	All recycled
3	1100	850	2.5-3.0	100	10.0	173.0	86.5	67.5	except sample
4	1100	1000	2.5-3.0	100	10.0	170.0	85.0	68.0	for anal.
5	1400	800	2.5-3.0	100	15.0	167.0	83.5	65.0	54-55
6	2000	1200	2.5-3.0	139	11.1	170.0	81.0	48.0	54-55
7	2000	1000	2.5-2.8	139	20.0	230.0	83.0	67.5	54-55
8	2000	1000	2.5-2.8	139	20.0	237.0	86.5	66.5	54-55
9	2000	1000	2.5-2.8	139	15.0	235.0	85.5	66.0	54-55
10	2000	1000	2.5-2.8	139	20.0	230.0	83.0	64.5	54-55
				139	20.0	230.0	83.0	64.5	54-55

*After pretreatment; recycling increases this value to 4.5-5.0.

†The highest theoretical yield is 77%.

low hydrogen ion concentration unfavorable to microbial activity. This is difficult to prevent when using caustic addition periodically as in other types of fermentation systems. Although a massive inoculum was applied, the continued increase in population was due, in large part, to the presence of the limestone in the system. The large surface areas for growing colonies of bacteria and the trace elements of the limestone (and probably Ca-ion, itself) were favorable toward propagation.

P. arabinosum is a representative member of the three genera of the family Propionibacteriaceae, Orla Jensen, 1909. Physical characteristics of *P. arabinosum* can be largely extended to other members of the family. It is expected that the behavior of the other genera would differ but slightly in optimum growth conditions. Choice of type was affected by this consideration, although there are other members of the family which produce volatile acids with more favorable ratio of HPr:HAC up to 5:1 (*P. thoenii*).

Optimum temperature described in the literature for various propionibacteria appears to be 30° C. The 35° temperature employed in the present investigation seemed to give satisfactory results. Higher temperature could be used as shown in some separate experiments in which strains of propionibacteria were acclimatized up to 50° C without influencing the metabolic activity.

There are several benefits realized from recycling fermented sulphite waste liquor. Returning neutralized liquor permits the use of fresh waste liquor of low pH; the calcium salts of organic acids interact with at least one portion of the ligno-acids present in the raw material. The recycling procedure utilizes not only the residual sugars but also the intermediate lactates, which are readily fermented to propionic acid. Lactates stimulate the production of propionic acid as found by other investigators (1).

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RELATIONS ENTRE LES PROPRIÉTÉS CYTOPATHOGÉNIQUES, HÉMADSORBANTES ET HÉMAGGLUTINANTES DU VIRUS DE L'INFLUENZA¹

A. BOUDREAU ET V. PAVILANIS

Abstract

Quantitative relations between hemadsorption, hemagglutination, and cytopathogenicity of influenza virus strains have been studied. The influenza virus has been cultivated on monkey kidney, chick kidney, and chick embryo cells.

It was shown that hemadsorption can also be used for titration of influenza virus adapted on tissue culture.

This technic is a reliable and sensitive one. It was also found that the growth of influenza virus is better in a medium of neutral pH and that chicks' age is not an important factor in the multiplication of the virus on chick kidney cells.

Introduction

Les techniques généralement utilisées pour titrer des suspensions de virus grippal adaptées à l'œuf de poule font appel à deux propriétés biologiques du virus: son infectivité pour l'œuf et son pouvoir d'agglutiner les globules rouges de coq. Or les souches grippales perdent graduellement ces deux propriétés si on les adapte à des cultures de tissus. Dans ce cas il est opportun de rechercher de nouvelles méthodes de titrage basées sur d'autres propriétés de ces virus.

Nous avons noté que les cultures de tissus infectées par différentes dilutions d'une suspension de virus grippal pouvaient adsorber les globules rouges de coq sans qu'on observe de production d'hémagglutinines.

Il nous a paru utile de comparer les titres de virus obtenus en utilisant la technique d'hémadsorption (AH) (4) avec ceux notés avec d'autres techniques comme l'hémagglutination, la cytopathogénicité et l'infectivité pour l'œuf. Incidemment nous avons été amenés à étudier l'influence du pH du milieu et de l'âge des poussins sur la multiplication du virus de l'influenza sur culture de tissus.

Matériaux et méthodes expérimentales

Culture de tissu

Trois cultures de cellules différentes, trypsinisées selon la technique de Bodian (1), ont servi à titrer le virus: rein de singe, rein de poussin, embryon de poulet.

Virus

Toutes les souches de virus utilisées ont été d'abord adaptées à l'œuf, puis aux cultures de tissus par un minimum de sept passages consécutifs.

¹Manuscrit reçu le 7 février 1961.

Contribution de l'École d'Hygiène et de l'Institut de Microbiologie et d'Hygiène de l'Université de Montréal, Montréal, Qué. Une partie de ce travail a été présentée au Congrès de la Canadian Public Health Association, section laboratoire, décembre 1960. Cette recherche a été partiellement subventionnée par le Ministère de la Santé Publique de Québec (subvention fédérale-provinciale à la recherche sur la Santé Publique).

TABLEAU I
Relation entre les propriétés hémasorbantes, cytopathogéniques et agglutinantes de virus grippaux

Souche	Temps (heures)	Embryon de poulet			Rein de poulet			Rein de singe			Oeuf
		Cyto log ₁₀	AH log ₁₀	Aggl. log ₁₀	Cyto log ₁₀	AH log ₁₀	Aggl. log ₁₀	Cyto log ₁₀	AH log ₁₀	Aggl. log ₁₀	EID/50 log ₁₀
PR8	24	0.0	4.0	2.0	0.0	4.7	3.0	0.0	5.6	2.0	5.5
	48	4.0	5.0	3.0	5.0	5.5	4.5	2.5	5.5	1.5	
	72	4.7	4.5	3.5	5.3	5.7	4.5	4.5	5.5	3.5	
	144	4.5	4.5	4.5	5.4	5.5	4.5	5.2	5.5	4.5	
BGL	24				0.0	2.5	0.0	0.0	2.5	0.0	4.0
	48				1.5	2.5	1.5	1.5	3.5	2.5	
	72				2.0	3.0	2.5	1.5	3.5	3.5	
	96				2.5	2.6	2.5	3.5	3.6	3.5	
	160				3.1	3.5	3.5	4.3	4.1	4.5	
PR8	24	0.0	4.2	0.0				0.0	4.7	0.0	4.7
	48	2.0	4.7	3.5				3.0	4.7	4.5	
	72	4.7	4.7	4.5				4.7	5.7	5.5	
	96	5.0	5.2	4.5				4.7	5.5	5.5	
R _{it} ⁺	24				0.0	1.5	0.0	0.0	1.5	0.0	4.5
	48				0.0	2.5	1.5	0.0	2.5	1.5	
	72				2.5	2.5	2.5	1.5	2.7	2.5	
	96				2.7	2.7	2.5	2.5	2.5	2.5	

Titrages

Le milieu M150 contenant 2% de sérum de veau a servi de diluant durant toute l'expérience.

Vingt tubes de culture de tissus ont été inoculés avec chaque dilution de virus. Quotidiennement, les tubes ont été examinés au microscope et le degré de cytopathogénicité a été noté. Les tubes où au moins 25% des cellules ont dégénéré ont été considérés positifs.

Chaque jour, durant 5 jours consécutifs, quatre tubes ont été pris au hasard dans chaque dilution. L'épreuve d'hémadsorption (AH) (4) a été exécutée sur le feuillet cellulaire de ces tubes avec des globules frais de coq, et l'épreuve d'hémagglutination (2) a été faite sur le liquide nutritif de ces mêmes tubes. Les tubes montrant un minimum de cinq zones d'hémadsorption ou un titre hémagglutinant minimum de 10 ont été considérés positifs.

L'épreuve de l'infektivité pour l'œuf a été faite de la façon usuelle en utilisant quatre œufs par dilution. La présence de virus a été recherchée par l'épreuve d'hémagglutination sur le liquide allantoïque de chaque œuf. Les dilutions donnant un titre agglutinant de 10 ou plus ont été considérées positives.

Tous les titres infectants ont été calculés d'après la méthode de Reed et Muench (3).

Résultats

(a) *Étude comparée des titrages par hémadsorption, cytopathogénicité, hémagglutination et infektivité pour l'œuf*

Après sept passages sur culture de tissu, les titres obtenus, avec chacune des trois techniques utilisées, sont voisins de ceux obtenus sur l'œuf (cf. tableau I). Il y a exception pour les souches asiatiques Ri₂ et Ri₄⁺,* mais avec ces mêmes souches, passées 13 fois sur rein de singe, nous avons obtenu récemment un titre hémadsorbant de 10^{-6.5} comparativement à un titre infectant pour l'œuf de 10^{-3.5}.

Le titre obtenu sur culture de tissu par la technique de l'hémadsorption est généralement plus élevé que par les deux autres techniques (Fig. 1 et Fig. 2). Ce fait s'accroît si les souches sont soumises à un plus grand nombre de passages sur ces cultures. La technique d'hémadsorption donne une réponse en 48 heures tout comme la technique de l'infektivité sur l'œuf.

L'effet cytopathogénique et l'apparition de particules virales hémagglutinantes sont plus tardifs, le titre maximum n'étant atteint qu'après 48 ou 72 heures alors que le titre maximum par la technique de l'hémadsorption est obtenu après 24 ou 48 heures.

(b) *Facteurs influençant la multiplication du virus de l'influenza sur culture de tissu*

(1) *pH du milieu*

Le liquide allantoïque des œufs inoculés avec le virus de l'influenza atteint un pH alcalin voisin de 8. Au contraire, sur culture de tissu, le pH reste à peu près neutre. Une expérience a été entreprise pour vérifier si un pH alcalin en culture de tissu ne donnerait pas des titres plus élevés.

*Nous remercions M. P. W. Choppin du Rockefeller Institute, N.Y., pour l'envoi des deux souches asiatiques Ri₂ et Ri₄.

Douze bouteilles de Roux dont une paroi était recouverte d'un feuillet de tissu de rein de singe ou de rein de poussin ont été ensemencées avec les souches PR8 et BGL adaptées à ces tissus par sept passages successifs. Une

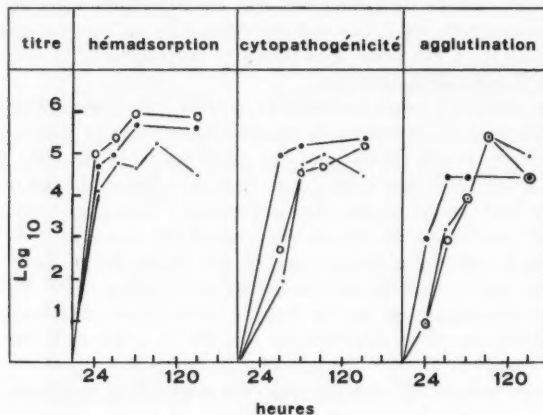


FIG. 1. Relations entre les propriétés hémasorbantes, cytopathogéniques et agglutinantes de souches grippales. ● Cellules d'embryon de poulet. ○ Cellules de rein de poulet. ○ Cellules de rein de singe. Souche A PR8.

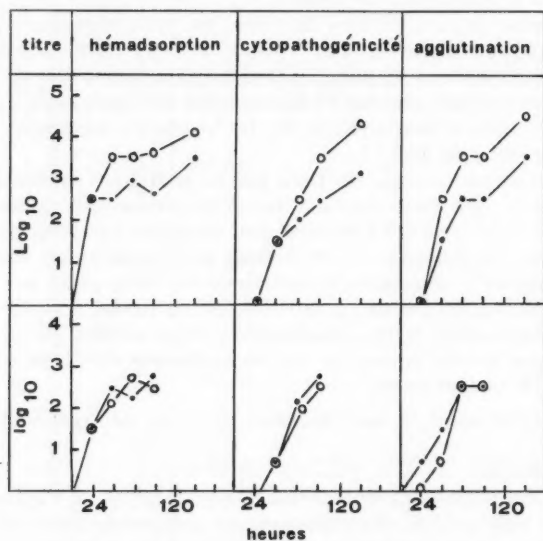


FIG. 2. Relations entre les propriétés hémasorbantes, cytopathogéniques et agglutinantes de souches grippales. ● Cellules d'embryon de poulet. ○ Cellules de rein de singe. Partie supérieure du graphique: souche BGL. Partie inférieure du graphique: souche asiatique No. 1751.

moitié des bouteilles contenaient 100 ml de milieu M150 et la deuxième moitié le même milieu porté à pH 8 par élimination du CO_2 . Un échantillon de 5 ml prélevé toutes les 24 heures a été titré sur des tubes contenant des cultures de cellules identiques pour rechercher les unités hémadsorbantes, cytopathogéniques et hémagglutinantes produites. Un volume égal de milieu frais a été ajouté pour remplacer l'échantillon prélevé.

Les résultats obtenus (Fig. 3 et Fig. 4) avec deux souches différentes, sur deux types de cellules, par trois techniques de titrages permettent de conclure que sur culture de tissu, de meilleurs titres sont obtenus avec un milieu dont le pH est voisin de la neutralité.

Les souches adaptées aux cultures de rein de singe donnent aussi des titres plus élevés que les souches adaptées aux cultures de rein de poussin. Ces résultats confirment également ce que nous avons déjà mentionné au sujet des relations entre les différentes techniques de titrage du virus grippal sur culture de tissu.

(2) Age des poussins

L'âge de l'hôte pouvant avoir une influence sur la multiplication du virus, nous avons étudié le degré de multiplication du virus de l'influenza sur des

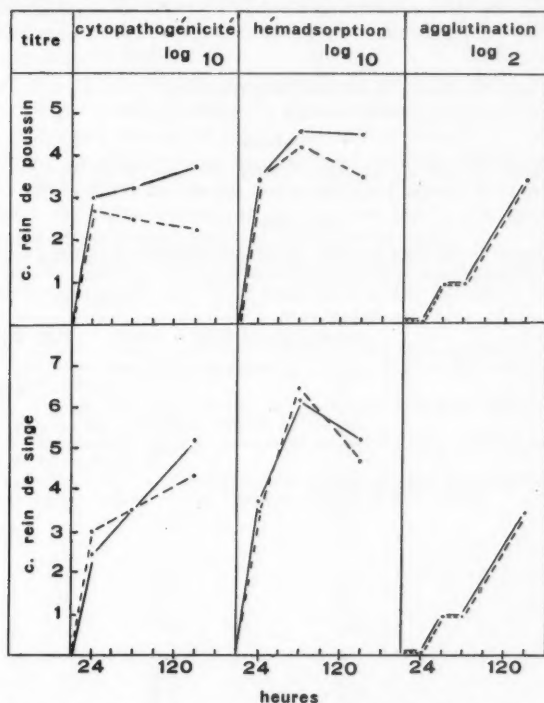


FIG. 3. Influence du pH sur la multiplication du virus de l'influenza en culture de tissu. Trait plein: pH neutre. Trait discontinu: pH alcalin. Souche A PR8.

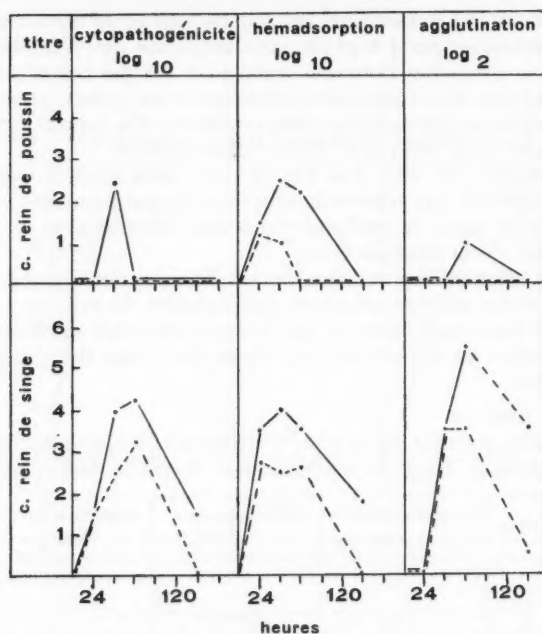


FIG. 4. Influence du pH sur la multiplication du virus de l'influenza en culture de tissu. Trait plein: pH neutre. Trait discontinu: pH alcalin. Souche BGL

TABLEAU II

Influence de l'âge des poussins sur la multiplication du virus grippal

Souche	Âge des poussins (jours)	Titre HA	
		Exp. 1 (\log_{10})	Exp. 2 (\log_{10})
PR8	1	2.0	
	2		5.5
	8	2.3	
	9		5.5
	16	1.5	
	18		3.5
	26		4.0
	32		5.5
	60	2.3	
Ri ₂	76		5.5
	1	1.3	
	8	0.2	
	16	1.0	
Ri ₄ ⁻	60	0.0	
	2	2.5	
	9	1.5	
	18	1.5	
	26	1.5	
	32	1.5	
	76	2.5	

cultures de reins prélevés de poussins d'âge différent.

Des reins prélevés de poussins âgés respectivement de 1, 8, 16, 26, 32, 60, 76 jours ont été trypsinisés et répartis en tubes. Après 6 jours, ces tubes ont été inoculés avec différentes dilutions de virus à raison de quatre tubes par dilution. Après 48 heures, le titre de virus produit a été recherché par la technique d'hémadsorption.

Il est évident d'après les résultats groupés dans le tableau II qu'il y a une plus grande différence entre deux expériences différentes répétées dans les mêmes conditions qu'entre les titres obtenus sur des reins provenant de poussins âgés de 1 à 76 jours. En aucun cas, la différence obtenue ne semble indiquer un âge optimum.

Discussion et conclusion

Les expériences décrites démontrent qu'il est possible d'utiliser les propriétés d'hémadsorption, de cytopathogénicité et d'agglutination du virus de l'influenza pour titrer les souches adaptées à des cultures de tissu.

Une technique de titrage basée sur l'hémadsorption est spécialement intéressante parce qu'elle donne une réponse en 48 heures et est facilement reproductible. Quand la souche dépasse un certain nombre de passages sur culture de tissu, cette technique devient plus sensible que la recherche du titre infectant pour l'œuf.

La technique de l'hémadsorption permet de plus un certain contrôle supplémentaire puisque l'examen à faible grossissement du feuillet cellulaire fait dans le but de repérer les zones d'hémadsorption permet en même temps de vérifier le genre de dégénérescence cellulaire qui est relativement typique pour le virus de l'influenza.

La simplicité de cette technique permet en outre son application dans tout laboratoire où se manipulent des cultures de tissu.

L'utilisation de cellules de rein de singe donne des titres plus élevés, même avec des souches n'ayant que deux passages sur ces cellules.

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IMMUNOLOGICAL STUDIES ON STAPHYLOCOCCAL PENICILLINASES¹

HARRIETT K. RHODES,² M. GOLDNER, AND R. J. WILSON

Abstract

Antistaphylococcal penicillinase sera were prepared using cell-free staphylococcal penicillinase concentrates as antigens in rabbits. Antisera to a purified *Bacillus cereus* penicillinase preparation were also prepared. Neutralizing antibodies were demonstrated in the immune sera by a microbiological assay method and precipitating antibodies demonstrated by agar diffusion.

A significant difference in the neutralizing ability of anti-*B. cereus* penicillinase serum against *Staphylococcus aureus* and *B. cereus* penicillinases was found. With the anti-*S. aureus* penicillinase serum, the difference was less marked. The neutralization titers of both antisera against *S. aureus* penicillinase were of the same order. Neutralization experiments suggested that there might be immunological differences in the penicillinases from different *S. aureus* strains.

Precipitation bands were obtained with purified preparations of staphylococcal and *B. cereus* penicillinases and homologous antisera, but no cross-precipitation was observed.

Introduction

The protection of penicillin from the action of staphylococcal penicillinase by anti-*Bacillus cereus* penicillinase serum has been demonstrated both in vitro (9) and in vivo (23). This cross-reaction with the staphylococcal enzyme is perhaps surprising since the penicillinases of *B. cereus* and *Bacillus subtilis* have been shown to be immunologically distinct (13). The possible therapeutic importance of the protective action of anti-*B. cereus* penicillinase serum in the treatment of infections with penicillin-resistant staphylococci has been examined by a number of authors (14, 15, 16, 2, 25). Similar studies using anti-staphylococcal penicillinase serum would be desirable since better protection would be expected.

The object of the present work was to prepare antistaphylococcal penicillinase sera and ascertain its ability to neutralize staphylococcal penicillinase. Difficulty has been experienced in obtaining antistaphylococcal penicillinase serum (20) and, on the basis of our experience and that of others (22, 24, 6) that the enzyme is intracellular, sonic lysates have been used as antigens. It was also proposed to make an immunological comparison of the penicillinases from *Staphylococcus aureus* and *B. cereus* and to investigate possible immunological differences between penicillinases from different strains of *S. aureus* (18).

Methods

PREPARATION OF STAPHYLOCOCCAL PENICILLINASE

Cell-free penicillinase concentrates were prepared from three strains of clini-

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cally isolated *S. aureus** following a method described in a separate paper (7). Twenty-four-hour broth cultures of the *S. aureus* strains were centrifuged and the washed bacterial sediment was suspended in sucrose-salt solution. The cells were then disrupted by subjecting the suspension to sonic vibrations and, after centrifugation at 17,000 g, the supernatant was dialyzed and lyophilized to yield a concentrate of staphylococcal penicillinase.

A purified staphylococcal penicillinase was also prepared. It was obtained by adsorption of the enzyme from the concentrate (Sask. No. 755) on fine-mesh powdered glass followed by elution and precipitation with ammonium sulphate solutions (1, 10). The product of the purification procedure was without perceptible penicillinase activity. When activated with reduced glutathione (0.1 M), it was found to be 50 times more active per mg nitrogen than the original concentrate.

Purified *B. cereus* NRRL-B569 penicillinase, 'Neutrapen', was kindly supplied by SchenLabs Pharmaceuticals, Inc.

PREPARATION OF ANTIPENICILLINASE

Preparation of Antigens for Injection

The penicillinase in 0.01 M phosphate buffer, pH 7.0, was sterilized by filtration through a Millipore filter (HA) in a Swinny adaptor (Millipore Filter Corporation, Watertown, Mass.).

To immunize with adjuvant (3, 4, 5), the antigen suspended in a 9:1 mixture of Bayol F and Arlacel A (21), with or without 4 mg/ml of dried heat-killed *Mycobacterium tuberculosis*, was used. For the injections, an emulsion was prepared containing 1 ml of penicillinase in the buffer and 1 ml of Freund's adjuvant.

Alum-precipitated antigen preparations were prepared as follows: the penicillinase in 0.75 ml of 0.01 M phosphate buffer, pH 7.0 (sterilized as described above), was mixed with 0.25 ml of 1% ammonium alum in the phosphate buffer. This was allowed to flocculate overnight in the cold and was resuspended before injection (18).

Immunization

Antisera against staphylococcal and *B. cereus* penicillinases were prepared in 2- to 3-kg rabbits, both with and without adjuvants. In the adjuvant series a modification of the procedure of Pollock (18) was followed while Tacking's method (23) was used for immunization without adjuvant.

Injections of antigen in Freund's adjuvant were made subcutaneously in the subscapular region, 1 ml on each side of the spine. Other antigen preparations were injected intravenously or intraperitoneally.

Actual amounts of antigen injected and times of administration are listed in the immunization schedule below. Desensitizing injections were given when there was danger of anaphylactic shock. Animals were bled out by cardiac puncture 7-10 days after the last injection.

*Sask. No. 755 (phage type 81), obtained from Dr. H. O. Dillenberg, Department of Public Health, Province of Saskatchewan, Regina, Sask.; H.S.C. No. 20 (phage type 6/7/53/77), obtained from Dr. T. E. Roy, Department of Bacteriology, Hospital for Sick Children, Toronto, Ont.; Windsor No. 1442 (phage type 81), obtained from Dr. D. M. Mills, Department of Pathology, Hôtel-Dieu of St. Joseph, Windsor, Ont.

Schedule

Method 1.—Neutrapen: two s.c. injections with an interval of 2 weeks, 17.7 mg (400,000 units, 5.6 mg N) per injection, with Freund's adjuvant with *M. tuberculosis*; after 2 weeks, five i.v. injections at intervals of 2–3 days, 7.7 mg (160,000 units, 2.2 mg N) per injection, alum precipitated. *S. aureus* penicillinase concentrate: four s.c. injections at intervals of 1 week, 50 mg (1850–7500 units, 3.2–5.1 mg N) per injection, with Freund's adjuvant (two injections with *M. tuberculosis*, two without); after 1–2 weeks, two to five i.v. injections at intervals of 2–4 days, 20 mg (1600–3000 units, 1.7–1.8 mg N) per injection, alum precipitated.

Method 2.—*S. aureus* penicillinase concentrate: 18 i.v. injections at intervals of 2–3 days: 3×5 mg (500 units, 0.55 mg N) + 6×10 mg (1000 units, 1.1 mg N) + 6×20 mg (2000 units, 2.2 mg N) + 3×25 mg (2500 units, 2.75 mg N) + one i.p. injection, 30 mg (3000 units, 3.3 mg N), at time of final i.v. injection.

DETERMINATION OF ANTIPENICILLINASE TITER

Antipenicillinase titers of the immune sera were determined in the following manner:

1. A neutralization mixture consisting of 0.2 ml of immune serum, either undiluted or diluted with normal serum, and 0.2 ml of the enzyme preparation in 1% gelatin was left at room temperature for 30 minutes. The gelatin was included to ensure stability of the penicillinase.
2. A 0.2-ml quantity of a solution of sodium benzyl penicillin in 1% phosphate buffer was added to the test mixture, which was then incubated for 60 minutes at 37° C to allow the unneutralized enzyme to act upon the penicillin.
3. At the end of 60 minutes, the tube containing the mixture was placed in a 75° C water bath for 5 minutes to inactivate the enzyme. The contents of the tube were then cooled rapidly in ice, frozen, and kept at -20° C until assayed. The same procedure was followed with a penicillin control and an enzyme control containing normal serum.
4. Assay of remaining substrate, penicillin, was carried out by means of a microbiological tube dilution method (1, 12). After test mixtures were thawed and sterilized by Millipore filtration, appropriate dilutions were made in broth*, and 0.5 ml of each dilution was added to an equal volume of a 1:10,000 dilution in the broth of a 24-hour culture of a sensitive *S. aureus* strain. The tubes were incubated at 37° C and read after 24 hours.

From the amount of penicillin remaining in the enzyme control series, the amount of enzyme originally present was calculated using the definition "One unit of penicillinase is that amount which is able to hydrolyze 1 unit of penicillin in 1 minute at 37° C and pH 7.0." This definition differs from that of Levy (11) only in specifying a temperature of 37° C instead of 25° C. By similar calculations from the results of the test series, the amount of penicillinase activity remaining after neutralization was determined. Then, using the defini-

*Medium 1, Assay Methods of Antibiotics, Antibiotic Monograph No. 2, Randall and Grove.

tion "One Neutralization Unit (N.U.) is that amount of antibody which neutralizes one unit of penicillinase in a mixture where enzyme is in excess" (18), the antipenicillinase titer of the immune serum was calculated and expressed in terms of neutralization units per ml of serum.

AGAR PRECIPITIN TEST

Precipitin tests in agar were performed using a modification of the Oudin technique (17, 19). Glass tubes 9-10 cm long with an inside diameter of 3 mm were used. The final concentration of agar in both serum and antigen layers was 0.3%. The tubes were filled 1/2 to 2/3 full with liquid serum-agar, which was allowed to harden. The serum-agar was then overlaid with liquid antigen-agar and the tubes were sealed. Bands were allowed to develop at room temperature over a period of 9-21 days. The intensity of each band was graded roughly by visual estimation.

Tests were done with a staphylococcal penicillinase concentrate and with the purified staphylococcal and *B. cereus* penicillinase preparations as antigens.

Results

In Table I are given the results of neutralization tests with *S. aureus* and *B. cereus* penicillinase preparations and their antisera. An immunological comparison of *B. cereus* and *S. aureus* penicillinases reveals a significant difference in the neutralizing ability of anti-*B. cereus* penicillinase serum against homologous and heterologous antigens. Wick, Holmes, and Boniece (25), using as a source of *S. aureus* penicillinase the supernatant from an overnight culture grown in the presence of penicillin, have made a similar observation in regard to the neutralizing ability of anti-*B. cereus* penicillinase serum. We observed that this marked difference in neutralization titers against homologous and heterologous antigens was not apparent with antiserum to *S. aureus* penicillinase. It should be noted in Table I that the neutralization titers of antisera to both *S. aureus* and *B. cereus* penicillinases were of the same order against *S. aureus* penicillinase.

TABLE I

Cross-neutralization tests with penicillinase preparations from *S. aureus* and *B. cereus*

	Serum No.	To strain	N.U./ml serum	
			vs. <i>S. aureus</i> penicillinase	vs. <i>B. cereus</i> penicillinase
Anti- <i>S. aureus</i> penicillinase	11	Sask. No. 755	1.03	2.1
	12	Sask. No. 755	0.08-1.03	—
	14	Windsor No. 1442	<u>0.61-1.31</u>	2.3
	15	Sask. No. 755	1.03	2.1
	19	H.S.C. No. 20	<0.06-1.31	0.34
Anti- <i>B. cereus</i> penicillinase	7	NRRL-B569	0.33	84
	8	NRRL-B569	1.03	84

NOTE: Underlining of numbers indicates "slightly greater than".

In Table II are given the results of neutralization tests with penicillinase preparations from different strains of *S. aureus* and their antisera. The cross-

PLATE I

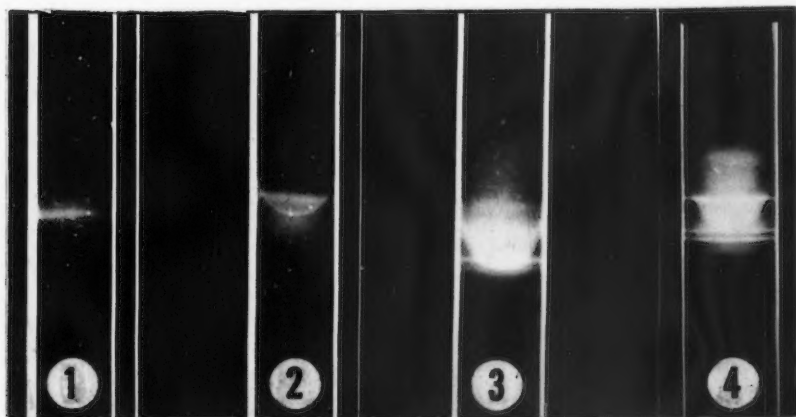


FIG. 1. Photographs of agar diffusion tubes described in Table III.

Rhodes *et al.*—Can. J. Microbiol.

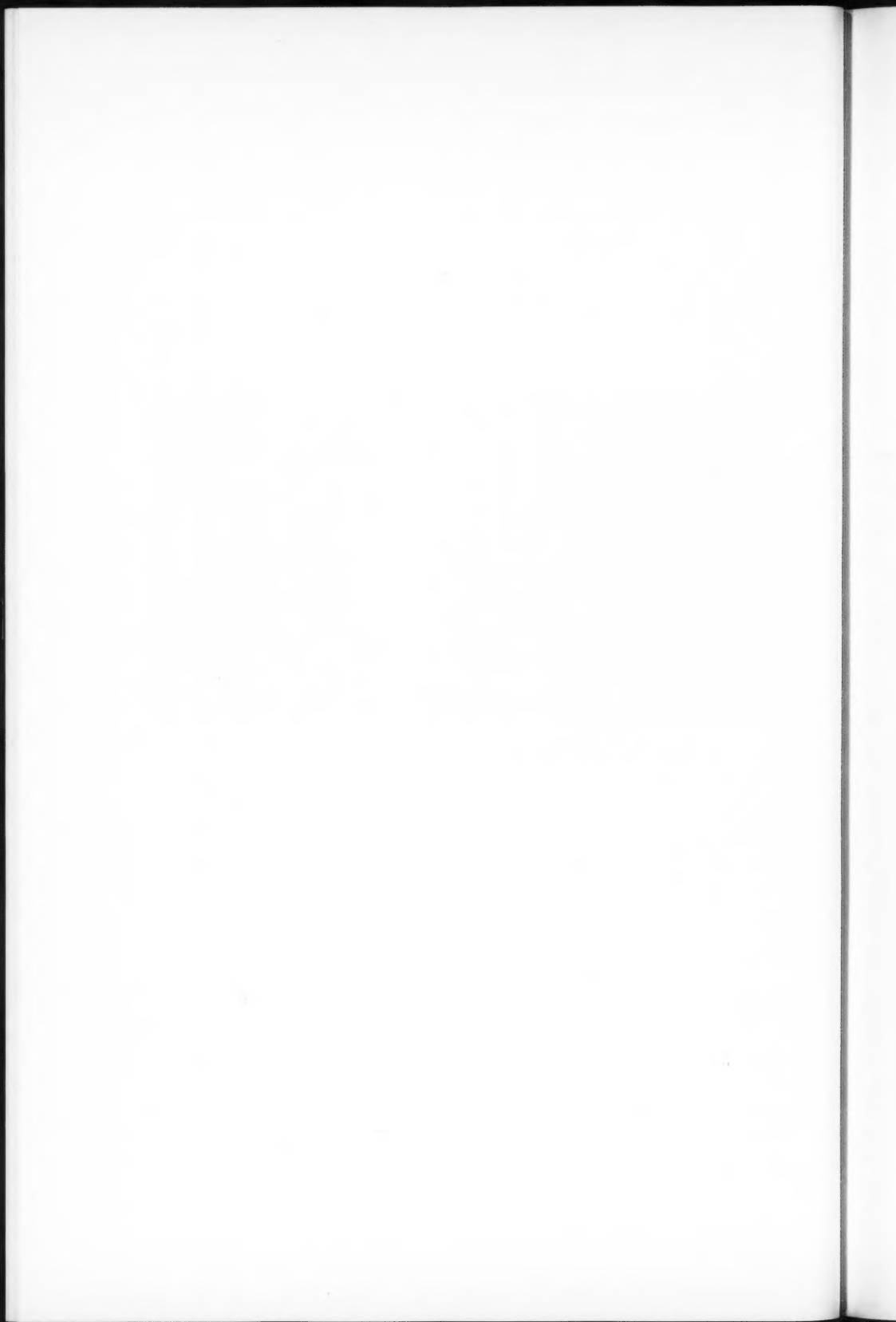


TABLE II

Cross-neutralization tests with penicillinase preparations from different strains of *S. aureus*

Serum	N.U./ml serum	
	vs. Sask. No. 755	vs. H.S.C. No. 20
Anti-Sask. No. 755	1.03	<u>0.08</u>
Anti-H.S.C. No. 20	<u>1.31</u>	<0.06
Anti-Windsor No. 1442	<u>0.61-1.03</u>	<u>1.31</u>

NOTE: Underlining of numbers indicates "slightly greater than".

reactions appear to indicate immunological differences between the preparations.

The use of adjuvants with *S. aureus* concentrates did not have any apparent effect on the level of neutralizing antibody.

[There is some indication that the loss of enzyme activity during purification does not affect the ability of the staphylococcal penicillinase to act as an immunizing agent. One assay of an antiserum to purified but non-activated *S. aureus* penicillinase gave a titer of slightly greater than 0.6 N.U./ml.]

The results of the precipitin analysis in agar of the antigen preparations are shown in Table III and Fig. 1. Tubes Nos. 1 and 2 were anti-Neutrapen serum with two different concentrations of Neutrapen. Tubes Nos. 3 and 4 were antistaphylococcal penicillinase serum with purified staphylococcal penicillinase and with staphylococcal penicillinase concentrate, respectively. The precipitin analyses indicate the minimum number of antigenic substances present in the penicillinase preparations.

More than one precipitation band was obtained with the two purified enzyme preparations from different bacterial sources and homologous antisera. However, the number of bands obtained was much lower than that obtained with the concentrate. Because the antigens in tubes Nos. 1, 2, and 3 were purified penicillinase preparations and the sera had been shown to contain antipenicillinase by neutralization tests, it is quite possible that one of the bands obtained with the homologous serum was formed by the penicillinase-antipenicillinase complex. However, identification of such a band has not yet been attempted.

The purified staphylococcal penicillinase preparation used in these tests had not been activated. If one of the bands was the result of penicillinase-antipenicillinase precipitation, it would indicate that the antigen need not be in the active state for precipitation to occur (8).

No cross-precipitation has been observed with purified *B. cereus* and *S. aureus* penicillinases and heterologous antisera.

Discussion and Conclusions

These experiments have shown that neutralizing antibodies against staphylococcal penicillinase can be obtained by immunization of rabbits with suitable enzyme preparations either with or without adjuvant. This is in contrast to

TABLE III
Agar precipitin bands using penicillinase antigens and antisera

Tube No.	Antiserum to:	Final concn.	Antigen	Source	State of purity	Final concn. per ml	Bands of precipitation *
1	Neutrapen	1:2	Neutrapen, <i>B. cereus</i> penicillinase	B569	Purified	1 mg	F, VF
2	Neutrapen	1:2	Neutrapen, <i>B. cereus</i> penicillinase	B569	Purified	2.5 mg	H, H, H
3	Windsor No. 1442	1:2	<i>S. aureus</i> penicillinase	Sask. No. 755	Purified	2.5 mg	F, H, F
4	Windsor No. 1442	1:2	<i>S. aureus</i> penicillinase	Sask. No. 755	Concentrate	12.5 mg	VF, F, VF, F, F, H ⁺ , H, H ⁺

*Intensity of bands is recorded as follows: H⁺, very heavy; H, heavy; F, faint; VF, very faint.

the results obtained by Prohaska and Zischka (20), who were unable to obtain antibodies using acetone-ether-treated cells as antigen. In the present work a concentrated sonic lysate of the organisms was used. Cross-neutralization tests with antisera and penicillinases from *S. aureus* and *B. cereus* showed that there were definite immunological differences between the penicillinases from these two bacterial species. In the case of cross-neutralization tests with penicillinases and antisera from different strains of *S. aureus*, it appeared that there might also be immunological differences between the enzymes from different staphylococcal strains.

Neutralization titers of antisera to *S. aureus* and *B. cereus* penicillinases were found to be similar against *S. aureus* penicillinase but not against *B. cereus* penicillinase. In vivo experiments by Tacking and others (23, 14, 15, 16, 25) showed that laboratory animals immunized against *B. cereus* penicillinase were thereby protected against the action of staphylococcal penicillinase during penicillin therapy. From the results shown in Table I, it would seem possible that this protection was effected by only a small proportion of the total anti-penicillinase content or neutralizing ability of the serum.

Three precipitation bands were obtained with a purified *S. aureus* penicillinase preparation and heterologous antistaphylococcal penicillinase serum. However, it was not possible to determine whether one of the bands actually was the penicillinase-antipenicillinase complex. There was no evidence of cross-reaction between any of the antigens in the purified penicillinase preparations from *B. cereus* and *S. aureus*. It is clear therefore that further immunological studies of penicillinases using gel diffusion techniques will require more highly purified enzyme preparations. This applies particularly to the analysis of penicillinases from different strains of *S. aureus*.

A proposed extension of these studies is the investigation of the possible utility of antistaphylococcal penicillinase sera in the therapy of infections with penicillin-resistant staphylococci.

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FORMATION OF TWO TYPES OF OSMOTICALLY FRAGILE BODIES FROM *STREPTOCOCCUS FAECALIS* VAR. *LIQUEFACIENS*¹

ARNOLD S. BLEIWEIS AND LEONARD N. ZIMMERMAN

Abstract

Osmotically fragile bodies which may be true protoplasts of the group D *Streptococcus faecalis* var. *liquefaciens* have been produced by use of a lytic enzyme derived from phage-infected cultures of the group D *S. faecalis* var. *zymogenes*. These bodies may be held in 1.1 M sucrose and are lysed almost quantitatively upon removal into water at room temperature or at 37° C. The phage-associated lysin, in the presence of 0.7 M cysteine, causes almost complete lysis of the sensitive organism by the end of 1 hour at 37° C and effects complete removal of cell-wall materials (rhamnose-containing moieties and phage-receptor sites) in that time.

Osmotically fragile bodies which may be termed spheroplasts of *S. faecalis* var. *liquefaciens* have been produced by the use of lysozyme on cells previously grown in penicillin (5 units/ml). Lysis to the extent of 50% in 1 hour at 37° C occurs in the aqueous suspensions, whereas 17% sucrose with 0.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ or 0.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ alone effects almost complete stabilization. The stabilized bodies are lysed almost quantitatively upon removal to water at 56° C. Cell-wall material containing rhamnose remains on these forms, indicating that they are spheroplasts rather than protoplasts.

Introduction

During the past few years much attention has been given to the use of protoplasts in genetic and physiological studies. Weibull (19) has reviewed the various methods of producing these osmotically fragile bodies as well as their potential value in research.

The streptococci, however, have proved to be refractory to the bacteriolytic effects of lysozyme, the enzyme which has been widely applied in the production of protoplasts of many Gram-positive organisms (15). As a result of this fact, production of these forms from streptococcal cells was not accomplished until recently when Freimer, Krause, and McCarty (7) reported conversion of group A strains using a phage-associated lysin obtained from a group C strain.

This paper describes the formation of two types of osmotically fragile forms of the group D *Streptococcus faecalis* var. *liquefaciens*, strain 31 by two different techniques: (a) lysozyme treatment of cells previously grown in penicillin and (b) application of a phage-associated lysin obtained from *S. faecalis* var. *zymogenes*.

Materials and Methods

Cultural Procedures

Two bacterial strains were used in this study: *S. faecalis* var. *liquefaciens*, strain 31 and *S. faecalis* var. *zymogenes*, strain 26C1a. The latter is a non-hemolytic mutant derived by ultraviolet irradiation from *S. faecalis* var.

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zymogenes, strain 26C1 (20). Stock cultures of these organisms were maintained in litmus milk at -17°C .

The two strains of bacteriophage that are reported here were isolated from local sewage samples. Each virus was brought up in an A-C broth culture of its specific bacterial host (the streptococcal strains mentioned in the preceding paragraph). Filtered lysates were stored at -17°C . Phage concentrations were determined by the conventional plaque-counting technique; plates were incubated for 20 hours.

Organisms were incubated at 37°C for all experiments. The media employed were A-C broth (14) or Penassay broth (Difco).

Cell inoculum was prepared as follows: A loop transfer was made from a stock culture which had been incubated for 24 hours, to a tube containing 10 ml of A-C broth. After 8 hours of incubation, the contents of the tube were added to 90 ml of A-C broth and incubated for an additional 5-6 hours. This cell suspension was then added to 900 ml of A-C broth and the whole incubated for 10-12 hours. Harvested cells were washed twice and resuspended in 10 ml of distilled water. All turbidity measurements were made using a 660 $m\mu$ filter with the Evelyn colorimeter.

Phage-associated Lysin Extraction Procedure

The lysin extract was prepared by modifying the procedure of Krause (11). An active culture of *S. faecalis* var. *zymogenes*, strain 26C1a, was brought up in A-C broth in a manner similar to that described for the cell inoculum; however, after incubation for 2.0 to 2.5 hours at the 1-liter level, phage was added in a concentration that would cause complete clearing of the suspension in 1-2 hours. The lysate was chilled, ammonium sulphate added to 0.6 saturation (0°C), and the mixture held for 3 days at 4°C . The precipitate formed was collected by centrifugation at $20,000\times g$ for 10 minutes at $8-10^{\circ}\text{C}$, resuspended in 50 ml of cold physiological saline, and recentrifuged to remove insoluble material. Ammonium sulphate (0.5 saturation, 0°C) was added to the supernatant and the mixture stored for 1.5 days at 4°C . The newly formed precipitate was removed (as above) and resuspended in 20 ml of cold 0.07 *M* phosphate buffer (pH 7.0); a final centrifugation removed the insoluble material. A dialysis was carried out for 18 hours at $8-10^{\circ}\text{C}$ against 25 volumes of physiological saline buffered at pH 7.0 with 0.07 *M* phosphate buffer. The final product (dialyzate) was slightly yellow and was stored at -17°C .

It was necessary to modify the above procedure, however, whenever lysin-treated cells were also analyzed for their rhamnose content because the yeast extract of A-C broth contains a rhamnose fraction. The interfering material was eliminated by substituting for yeast extract the salts, purines, pyrimidines, and vitamins of the casein semisynthetic medium (CSM) of Rabin and Zimmerman (14). The remainder of the extraction procedure remained unchanged.

Miscellaneous Materials of Importance

The following crystalline compounds of importance were used: penicillin G (potassium) (Nutritional Biochemicals Corporation); lysozyme (Armour and Company, and Worthington Biochemical Corporation); and catalase (General Biochemicals, Inc.).

Results

Osmotically Fragile Forms Using Penicillin and Lysozyme in Combination

1. Bacteriolysis by Penicillin

Lederberg (12) has reported the formation of osmotically fragile forms of *Escherichia coli* with penicillin (1000 units per ml). It has since become evident that Gram-positive cells are more sensitive to the antibiotic than Gram-negative organisms because of their possession of certain cell-wall moieties whose incorporation into the cell wall is inhibited by penicillin (13, 18). Preliminary experiments were designed to study the effects of varying penicillin concentrations and of varying cell concentrations on lysis of the Gram-positive *Streptococcus faecalis* var. *liquefaciens*. The first experiment of this series showed that 1000 units of penicillin per ml of Penassay broth would not lyse cells in concentrations varying in initial optical density from 0.191 to 0.509. There was instead a uniform increase in turbidity within a 2-hour period before the cessation of growth; no further optical density changes occurred after an additional 2 hours. Similarly, with the use of a constant initial cell concentration (optical density readings ranging from 0.305 to 0.315) and of penicillin concentrations varying from 0 to 1000 units per ml of Penassay broth, there was a fairly uniform increase in turbidity within a period of 2.5 hours. It thus was shown that this streptococcus, which is more sensitive to the bactericidal effects of penicillin than is *E. coli*, is refractory to the bacteriolytic effects of the antibiotic in a system similar to that employed by Lederberg (12) in his studies with the latter organism.

2. Bacteriolysis by Lysozyme

Although lysozyme is known to be ineffective as a lytic agent for streptococci (15), the data of Fig. 1 show that prior treatment with penicillin makes this streptococcus more susceptible to lysis. For instance, lysozyme (16 μ g per ml)

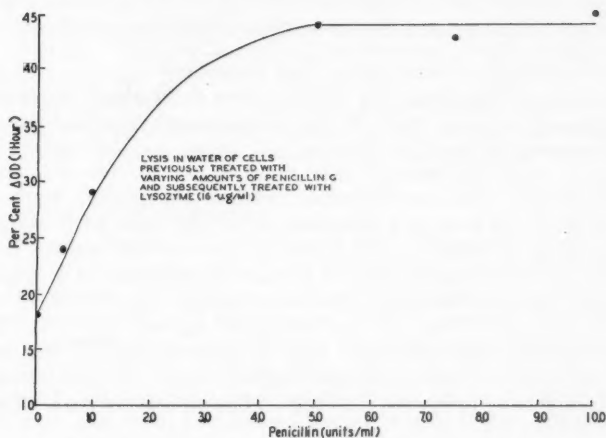


FIG. 1. The effect of lysozyme on cells previously treated with various amounts of penicillin. Initial optical density readings range from 0.295 to 0.312.

caused only 18% lysis of untreated cells in 1 hour but 45% lysis in 1 hour (80% lysis in 4 hours (not shown in Fig. 1)) of cells previously grown in Penassay broth containing 10 units of penicillin per ml. Penicillin concentrations below 5 units per ml produced subsequent lysis with lysozyme to lesser degrees. This effect was used to produce the lysozyme-sensitive organisms for future experiments by inoculating cells (initial optical density 0.300–0.350) in Penassay broth containing 5 units of penicillin per ml. After 2 hours of incubation, the cells were removed by centrifugation and resuspended in the same volume of a solution containing lysozyme and buffer.

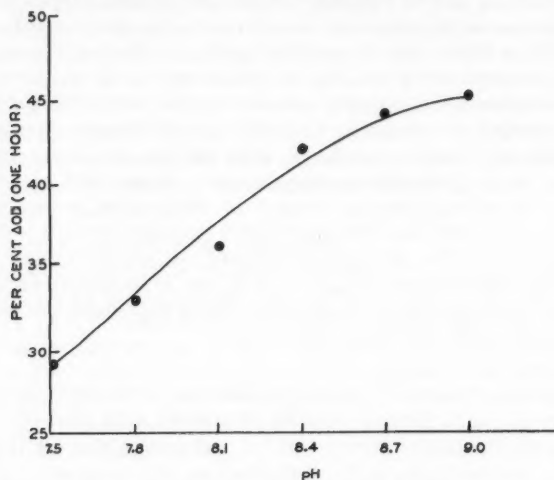


FIG. 2. Effect of pH on lysis of penicillin-treated cells by lysozyme.

3. Optimal Lysozyme Concentrations and Conditions

The optimal pH for lysozyme activity was determined by resuspending penicillin-treated cells in 0.03 *M* tris(hydroxymethyl)aminomethane (Tris buffer) previously adjusted to various pH levels and by adding lysozyme (Worthington) to a final concentration of 16 μ g per ml. The results shown in Fig. 2 indicate that alkaline conditions produce the greatest optical density changes. A pH of 8.4 was used, therefore, in all the work with lysozyme.

Whereas 16 μ g of Worthington lysozyme per ml was sufficient to give a 45–50% change in optical density in 1 hour, 40 μ g of Armour lysozyme was required to produce the same optical density change. Figure 3 shows the results of a plot of lysozyme (Armour) concentration against cell lysis. This figure also demonstrates that relatively high concentrations of enzyme caused apparent decreases in lysis, perhaps resulting from the formation of large complexes of the basic lysozyme with the acidic polymers in the medium (RNA, DNA, etc. (16)).

4. Stabilization of Osmotic Pressure to Prevent Lysis

Lederberg (12) reported that 20% sucrose and 0.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ effected

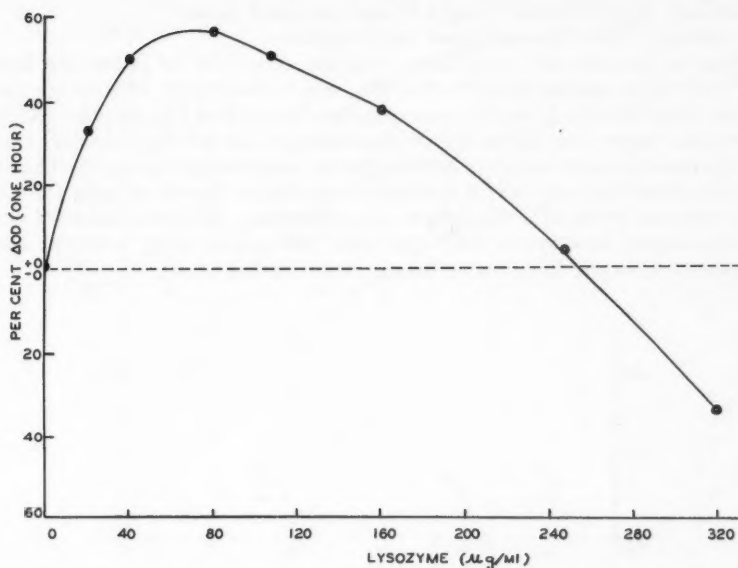


FIG. 3. Effect of varying lysozyme concentrations on penicillin-treated cells.

complete stabilization of the osmotically fragile forms produced by his penicillin treatment. This system (using 17% sucrose) was adopted for use in these studies and resulted in almost complete stabilization of the fragile bodies. Sucrose alone was completely ineffective but $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2%) alone allowed only 10% lysis during the first hour although, by the end of 2 hours, 25 to 30% lysis had occurred. The combination of sucrose and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was most effective over the longer time (only 10% lysis).

Ordinarily, resuspension of the stabilized bodies in distilled water at 37° C for 1 hour resulted in incomplete lysis; however, when these bodies were incubated at 56° C (a procedure employed by Hurwitz *et al.* (10)), complete lysis occurred.

5. Analysis for Cell Wall Removal

An important criterion for confirming the notion that these stabilized bodies represent true protoplasts (2) is the absence of cell wall material. In this case, the absence of rhamnose, a major constituent of streptococcal cell walls, would be indicative of the success of the treatment. The Dische and Shettles (5) methylpentose assay (using the 10-minute boiling time recommended by Dische (4)) was used to make this determination. Analyses from samples of untreated cells, stabilized bodies, and the supernatant and sediment from centrifuged portions of stabilized bodies, revealed that over 90% of the sugar had remained on the treated cells. The osmotically fragile bodies produced by the penicillin and lysozyme treatment were therefore considered to be spheroplasts (10) rather than protoplasts.

*Osmotically Fragile Forms Using a Phage-associated Lysin**1. Optimal Lysin Concentrations and Conditions*

When *S. faecalis* var. *liquefaciens*, suspended in 0.07 *M* phosphate buffer (pH 7.0) at an optical density of 0.400, was treated with 10% of the lysin extract, there was little lysis (compare curves A and B of Fig. 4). The addition of cysteine hydrochloride to a final concentration of 0.7 *M*, however, led to the rapid and almost complete lysis of the cell suspension (curve D). That the cysteine alone was capable of causing a significant degree of lysis (35%) is demonstrated in curve C. Complete lysis, therefore, should be regarded as an interdependent function of both the lysin and amino acid, with the latter perhaps functioning to a certain extent as a reducing agent.

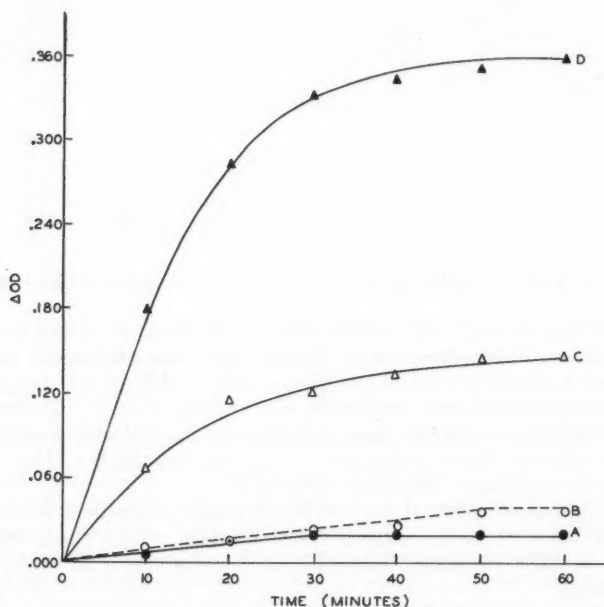


FIG. 4. Comparative rates of lysis caused by lysin, cysteine, and combination of both: A, untreated cells; B, lysin (10%) treated cells; C, cysteine (0.7 *M*) treated cells; D, lysin (10%) and cysteine (0.7 *M*) treated cells.

Rate studies using varying amounts of lysin in 0.7 *M* cysteine proved that 10% extract was adequate in accomplishing complete lysis in 25–30 minutes. Results from similar studies using 10% lysin but varying cysteine concentrations can be seen in Fig. 5, which shows that the final amount of lysis is proportional to the cysteine concentration but that the initial rate of lysis (i.e., first 10 minutes) is independent of the concentration from 0.4 to 0.7 *M*. These data are a further indication of the dual role played by the amino acid.

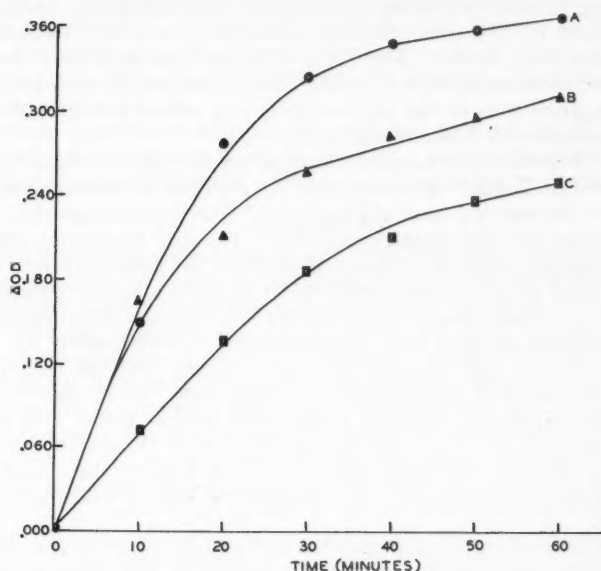


FIG. 5. Determination of optimal cysteine concentration: A, 0.7 M cysteine-HCl and 10% lysin; B, 0.4 M cysteine-HCl and 10% lysin; C, 0.1 M cysteine-HCl and 10% lysin.

2. Stabilization of Osmotic Pressure to Prevent Lysis

Freimer *et al.* (6) reported that 0.6–0.9 M sucrose was effective for osmotic stabilization of their group A streptococcal protoplasts; however, 1.1 M sucrose was found necessary to obtain minimal lysis (15% in 1 hour) with the group D streptococcus used in this study. The ability of the bodies to be held in a stabilizer with no disruption of cell membranes excluded the possibility of contaminating proteolytic enzymes in the polysaccharase extract.

But the possibility of interference with the treatment by the sucrose could not be disregarded. Accordingly, stabilized treated suspensions were centrifuged at $20,000\times g$ for 20 minutes and were resuspended in water. Immediate osmotic lysis, at room temperature, occurred to the extent of 55% of the original concentration; further incubation at 37° C led finally to almost complete lysis. These results indicate, therefore, no interference with the treatment by the stabilizer and also demonstrate the extreme fragility of these stabilizable bodies as compared to those produced by the penicillin-lysozyme treatment.

3. Analysis for Absence of Cell Walls on Osmotically Fragile Bodies

To determine the cell wall residuum on the fragile bodies, the Dische and Shettles methylpentose assay was applied again. The presence of the sucrose stabilizer, which charred upon boiling, and the cysteine, which interfered with the color reaction, necessitated the adoption of an alternate procedure; to this end, a modification of a method by Slade and Slamp (17) was used. Briefly,

the technique involved removing, at 10-minute intervals during 1 hour, samples of unstabilized, lysin-treated cells and adding H_2O_2 to a final concentration of 1.4% to stop the reaction. (The 1.4% H_2O_2 had the additional function of oxidizing the excess cysteine to cystine.) The cystine precipitates were removed by centrifugation and to the supernatants was added enough catalase (100 $\mu g/ml$) to completely dissipate the excess H_2O_2 .

In Fig. 6 observations are depicted comparing the rates of rhamnose removal and cell lysis. It is quite apparent that the removal of the sugar was almost complete by the end of 1 hour although total lysis lagged somewhat. The rate of lysis, though initially greater than that of cell wall dissolution, fell sharply at 25–30 minutes, while rhamnose continued to be released at only a slightly lower rate until almost entirely removed. (Complete removal required 3 hours of incubation.) These observations and the fact that the rhamnose-containing cell-wall material released from treated cells is non-dialyzable (for a similar finding see Gooder and Maxted (8)) may indicate that the cells had burst before all the cell wall was removed. Perhaps some "weak spot" exists that was denuded of cell wall material before other surface areas. This conclusion was also reached by Slade and Slamp (17), who found the same general relationship between rhamnose release and cell lysis in their studies of the effects on group A streptococci of a bacteriolytic enzyme liberated by *Streptomyces albus*.

The final series of experiments related to the extent of cell-wall removal from the stabilized, lysin-treated cells were concerned with demonstrating the absence of phage receptors. Equivalent phage was added to tubes of treated and untreated cells suspended in 1.1 M sucrose, the concentration of phage being selected to provide an excess of receptors (cell to phage ratio being 7:2).

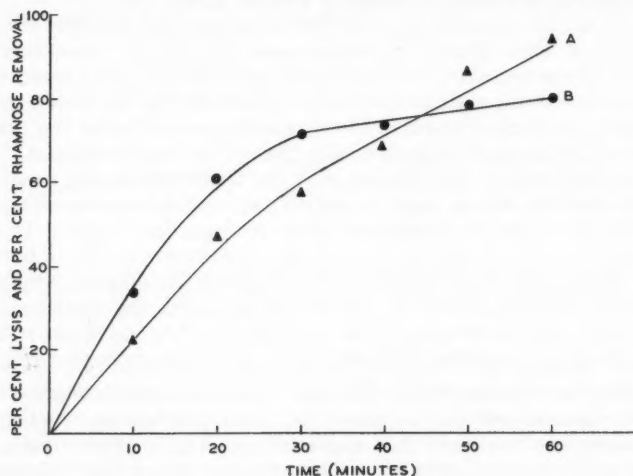


FIG. 6. Comparison of rate of rhamnose removal to rate of cell lysis: A, rhamnose removal; B, lysis.

After 1 hour of incubation at 37° C to allow adsorption of the virus, the suspensions were centrifuged and the supernatants assayed. The results showed that no phage was taken up by the fragile bodies, compared to at least 95% adsorption by the whole cells; the data indicated the absence of phage receptors on the lysin-treated cells.

From these experiments showing the apparent removal of cell walls, and from turbidimetric studies, it seems that the phage-associated lysin can convert almost quantitatively *S. faecalis* var. *liquefaciens* to protoplasts.

Discussion

As shown previously, pretreatment with penicillin significantly increases the lysozyme-sensitivity of *S. faecalis* var. *liquefaciens*. Studies by Brumfitt *et al.* (3) on the cell walls of *Micrococcus lysodeikticus* led these investigators to conclude that the lysozyme-susceptible linkage is the β 1-4 bond between N-acetylmuramic acid and N-acetylglucosamine. Recent studies by Hayashi and Barkulis (9) indicate the absence of such a bond in streptococcal cell walls. These authors have suggested that the N-acetylmuramic acid moiety is linked instead to the rhamnose polysaccharide. Assuming the hypothesis of Brumfitt *et al.* (3) is correct, the normal lysozyme-resistance of most streptococcal strains can be thus explained. The presence of other sugars and of O-acetyl groups, which have been shown to be present in cell walls of *Streptococcus faecalis* by Abrams (1), at the sensitive site would also confer resistance to lysozyme upon an organism. Following this line of reasoning, it would appear that pretreatment with penicillin causes a rearrangement of the cell wall such that lysozyme-sensitive linkages are created or, possibly, such that already existing linkages are made more available (exposed) to the lytic enzyme.

The results which have been presented herein, however, indicate that conferral of lysozyme-sensitivity by pretreatment with the antibiotic does not lead to complete removal of cell-wall material, even though stabilizable, osmotically fragile forms are produced. The rhamnose-containing moieties of the wall remain on the cell, although possibly other substances are liberated. It would be interesting, in this regard, to determine if N-acetylglucosamine is liberated upon lysozyme treatment.

The phage-associated lysin, on the other hand, may remove all the cell wall from the organism. Rhamnose-containing moieties as well as phage-receptor sites were shown to be completely removed from the cell. In addition, the stabilized forms produced by such treatment were more osmotically fragile than those produced by the penicillin-lysozyme treatment. It is obvious, therefore, that the substrates of the two lytic preparations employed are not the same and that the lysin substrate is far more available.

That the utilization of phage-associated lysins for the production of protoplasts can be an important new technique for many organisms is clearly implied by the work reported herein. Earlier findings by Freimer *et al.* (7) have shown the group A streptococci to be susceptible to an appropriate lysin. The work reported herein introduces the technique to the group D streptococci. It is conceivable that any organism susceptible to a phage may produce these autolytic enzymes which can then be applied to this same organism or to closely related organisms for the production of protoplasts. Indeed, such

diverse bacterial forms as *Escherichia*, *Staphylococcus*, *Klebsiella*, and *Bacillus megaterium* are known to liberate lytic enzymes upon phage infection (11). These enzymes may also have taxonomic applications. Their use in this area is implied by successful application of the lysins to organisms other than the strain that produced the enzyme as demonstrated by Gooder and Maxted (8) and in this paper.

It can be concluded that whereas the combined penicillin and lysozyme treatment results in the formation of spheroplasts, the use of specific phage-associated lysin in the presence of 0.7 M cysteine results in the formation of bodies devoid of cell-wall material. Because these forms have not been tested for all the characteristics of true protoplasts as set forth by Brenner *et al.* (2), they cannot yet be definitely so designated. However, since the important criterion of the absence of cell-wall material is satisfied, these bodies can be tentatively termed protoplasts of *S. faecalis* var. *liquefaciens*.

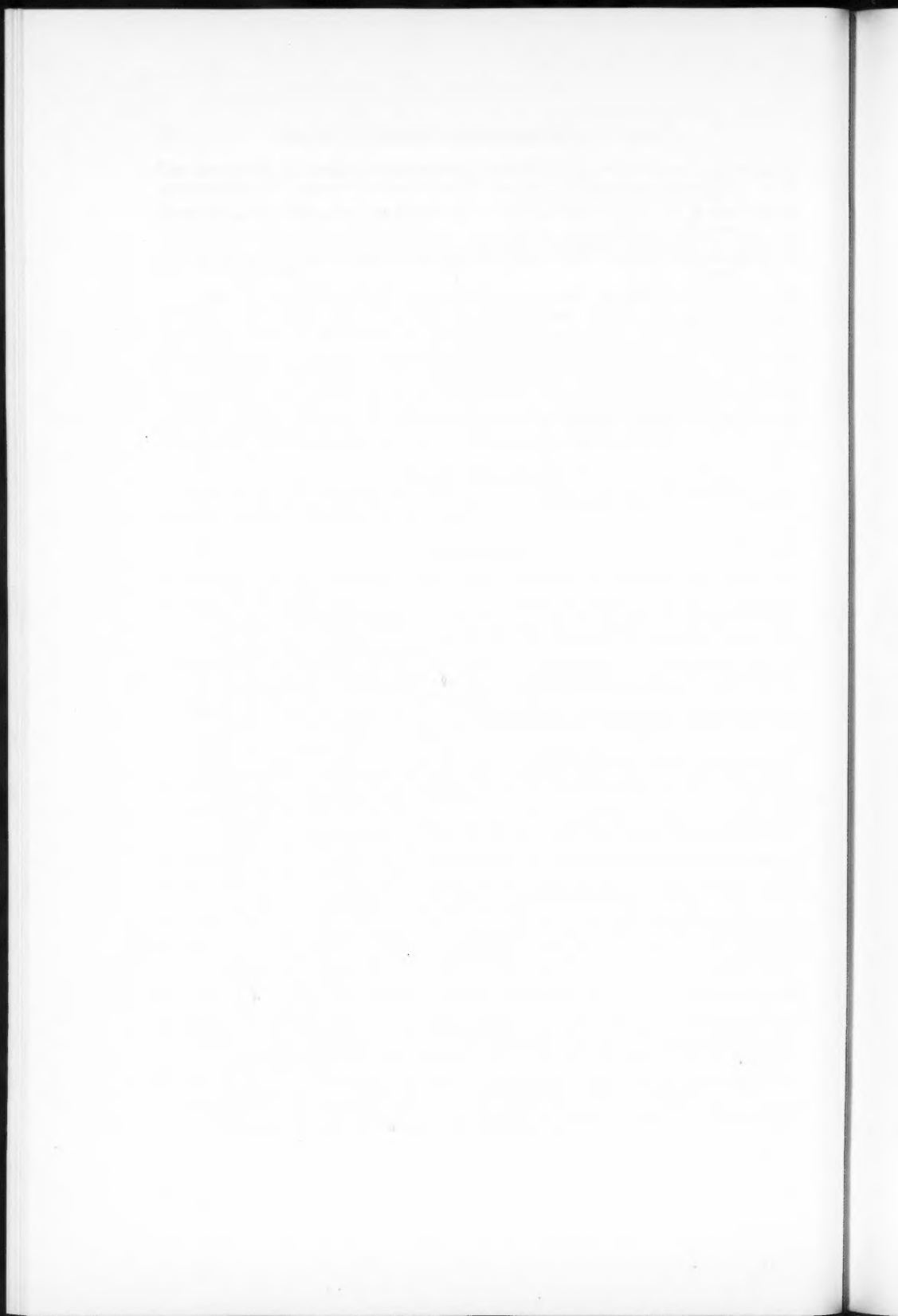
Acknowledgment

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BACTERIOLOGICAL STUDIES OF FRESH-WATER FISH

I. ISOLATION OF AEROBIC BACTERIA FROM SEVERAL SPECIES OF ONTARIO FISH¹

T. P. T. EVELYN² AND L. A. McDERMOTT

Abstract

A total of 350 fish were taken from Ontario waters and examined for the presence of aerobic bacteria. Trout species accounted for 97.1% of the fish examined, and the remainder were "coarse" fish. A total of 1189 cultures of bacteria were isolated from hearts, livers, kidneys, spleens, muscles, and slime of the fish and from 11 of the waters they inhabited. Of this total, 221 cultures were lost through death, most of these organisms being too fastidious to survive the routine cultural methods employed. Of the remaining 968 cultures, 121 proved impossible to identify or group on the basis of common characteristics. The remaining bacteria were classified to the rank of genus as follows: 234 *Pseudomonas*, 140 *Aeromonas*, 111 *Micrococcus*, 96 *Lactobacillus*, 72 *Escherichia*, 57 *Brevibacterium*, 29 *Paracolobacterium*, 28 *Aerobacter*, 24 *Proteus* and other urea splitters, 18 *Alcaligenes*, 14 *Bacillus*, 11 *Achromobacter*, 7 *Streptococcus*, and 6 *Flavobacterium*.

Introduction

Bacteria are known to be responsible for several important fish diseases. During routine examinations of fish for bacterial infections in this laboratory, it was soon apparent that very little was known of their associated bacteria, pathogenic or otherwise. Accordingly, this study was initiated to determine the nature of the aerobic bacterial flora associated with several species of Ontario fresh-water fish. The investigation was made possible by the co-operation of and financial aid from the Ontario Department of Lands and Forests and the National Research Council of Canada.

Investigations of the bacterial flora of fish have been confined largely to those associated with marine fish. Moreover, the emphasis in these studies has been placed on those microorganisms associated with fish spoilage. Griffith (15) and Tarr (40) have reviewed the literature on the microbiology of marine fish. While the generic distribution of the bacteria associated with freshly caught marine fish is highly variable, the following genera are reported to predominate fairly consistently: *Pseudomonas*, *Achromobacter*, *Flavobacterium*, and *Micrococcus*. The genera *Proteus*, *Sarcina*, *Bacillus*, *Corynebacterium*, *Serratia*, and *Vibrio* are encountered less often.

Comparatively little attention has been paid to the bacterial flora of fresh-water fish. Thjøtta and Sømme (41) isolated 115 strains of bacteria from the skins, gills, and intestines of 11 fresh-water fish. They found the members of the following genera of bacteria present in order of decreasing numbers: *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Chromobacterium*, *Micrococcus*,

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Escherichia, *Salmonella*, *Shigella*, and *Proteus*. In addition, a number of questionable species were present.

More recently, Venkataraman and Sreenivasan (43) isolated 95 strains of bacteria from the slime, flesh, and peritoneal cavities of four species of fresh-water fish. They found the following genera of bacteria in decreasing order of numerical importance: *Micrococcus*, *Flavobacterium*, *Paracolobactrum*, *Achromobacter*, *Bacterium*, *Alcaligenes*, *Pseudomonas*, *Bacillus*, *Sarcina*, *Aerobacter*, and *Microbacterium*. These workers experienced difficulty in classifying their isolates and described one rod and four coccus forms which they believed to be new species.

Other bacterial surveys of fresh-water fish have been largely interested in the presence of the coli-aerogenes and salmonella-shigella groups of bacteria in the digestive organs (1, 12, 42).

Most of the existing knowledge of the bacteria associated with fresh-water fish results from investigations of fish infections. A number of fish diseases are caused by bacteria belonging to the *Aeromonas* and *Pseudomonas* genera (37). *Aeromonas salmonicida* (Lehmann and Neumann) Griffin is the causative agent of furunculosis, a generalized septicemic disease of salmonid and other fish (21, 35, 36). *Aeromonas hydrophila* (Chester) Stanier causes "red sore disease" in pike, trout, and other fish (13, 19, 27, 39, 44). *Aeromonas punctata* (Zimmermann emend, Lehmann and Neumann) Snieszko is associated with diseases in carp (33). Wolf (45) reported an air bladder disease in lake trout fingerlings caused by *Pseudomonas fluorescens* Migula. Seaman (31) blamed a species of *Pseudomonas* as the cause of a disease in hatchery rainbow trout, and Rucker *et al.* (30) included species of *Pseudomonas* as causing infectious diseases of Pacific salmon.

Brisou *et al.* (5, 6) isolated three species of bacteria from fresh-water fish. Two of the bacterial cultures were identified as species of the genus *Flavobacterium* and one of the genus *Achromobacter*. These same workers (7) isolated a pathogenic species of bacteria from trout and suggested it be named *Erwinia salmonis*. *Haemophilus piscium* Snieszko *et al.* is the causative agent of ulcer disease in fish (25, 34). Another disease of fish, known as kidney disease, and thought to be caused by a species of *Corynebacterium* (23), is assuming a role of major importance (9, 38, 46).

Flakas (11) isolated a bacterium from diseased black crappies which he tentatively placed in the genus *Spherotheca*. Margolis (20) isolated a bacterium from a diseased white sucker which appeared to belong to the genus *Paracolobactrum*. Griffin and Snieszko (14) reported an organism similar to *Paracolobactrum aerogenoides* Bordman *et al.* as being pathogenic to fish. Infections with species of *Vibrio* are briefly reviewed by Rucker (29), while Parisot (24) has reviewed mycobacterial infections in marine and fresh-water fish. Rucker *et al.* (30) have discussed the importance of myxobacteria associated with fish diseases.

Materials and Methods

Fifty catches of fish were taken from 43 areas in the province of Ontario. A total of 350 fish were examined; 129 from hatcheries, 185 from streams and rivers, 14 from lakes, and 22 from ponds.

Bacteriological examinations were conducted as soon as possible after the fish were caught so that the bacteria isolated would presumably represent the bacterial flora during life. When it was obvious that the interval between catching and bacteriological examination in the laboratory would be longer than one day, the fish were examined in the field using portable equipment. However, in field trips involving only short distances, the fish were placed in cellophane bags in a portable ice chest or kept alive in tanks and examined immediately upon return to the laboratory. In a few instances, fish were received at the laboratory in a solidly frozen state, and these were also examined if they were in a satisfactory condition.

Sampling was largely restricted to the trout species since trout are reared in all the hatcheries visited and are susceptible to many bacterial infections. The fish were taken with dip nets at the hatcheries. Where larger bodies of water were sampled, such as lakes and ponds, seines were employed. A portable electric shocker was usually employed in such natural habitats as streams, the stunned fish being caught with dip nets before they could revive.

The survey was limited to the isolation of aerobic bacteria, especially those associated with the heart, liver, and kidney. A few isolations were also made from abnormal spleen and muscle tissues. It was planned to examine the waters of the areas from which the fish were taken and also the slime of the fish. However, both of these procedures proved to be too time-consuming and were discontinued early in this investigation.

Details of the procedures followed in isolating the bacteria from the fish and in identifying each isolate are described by Evelyn (10). The bacteria were classified according to the system outlined in Bergey's Manual of Determinative Bacteriology (3). All tests were conducted under aerobic conditions only, and no studies were made of the antigenic structures or serological relationships of the bacteria isolated.

Results and Discussion

The numbers of fish examined bacteriologically during this survey are shown in Table I. The emphasis in sampling was placed on the trout species, and of these, brook and brown trout were usually encountered more frequently

TABLE I
Numbers of fish examined during the period June 2, 1958 - December 3, 1959

Species of fish	Numbers examined
Brook trout (<i>Salvelinus fontinalis</i> Mitchill)	151
Rainbow trout (<i>Salmo gairdnerii irideus</i> Gibbons)	79
Brown trout (<i>Salmo trutta fario</i> Linnaeus)	69
Lake trout (<i>Salvelinus namaycush namaycush</i> Walbaum)	37
Splake	4
Brown bullhead (<i>Ictalurus nebulosus nebulosus</i> LeSueur)	4
Creek chub (<i>Semotilus atromaculatus atromaculatus</i> Mitchill)	2
Common sucker (<i>Catostomus commersonnii commersonnii</i> Lacepede)	2
Common shiner (<i>Notropis cornutus</i> Mitchill)	1
Sunfish (<i>Lepomis</i> sp.)	1
Total	350

than rainbow trout in the areas sampled, although a greater total of rainbow trout were caught than brown trout. The lake trout examined were largely hatchery fish. The splake, a hybrid resulting from a brook trout and lake trout cross, is still an experimental fish so that few of them were available for examination. Most of the fish appeared healthy, although the four splake bore large, red, external lesions and four of the brook trout had extensive dorsal lesions suggesting mechanical injury. Twenty-seven of the lake trout from hatcheries showed loss of appetite, and five moribund brook trout taken from natural streams were infected with furunculosis.

A total of 1189 bacterial cultures were isolated from the fish, and 221 of these were lost. The majority of the latter were fastidious types which appeared as pinpoint colonies on primary streak plates. Most of them failed to grow after one transfer or after storage at 4° C, and it was apparent that the routine cultural methods employed were not satisfactory for their maintenance. Of the remaining 968 cultures of bacteria, 121 proved to be a heterogeneous lot of individuals. Not only was it impossible to identify them as being related to any known bacterial types, but it proved impossible to obtain any coherent groupings based on common characteristics. The remaining 847 cultures of bacteria formed groups which were recognizable.

It is not possible to state precisely the actual site or sites occupied by the bacteria isolated from the internal organs of the fish examined. The bacteria could have been situated within the tissues examined, or, conceivably, could have been present in the body cavity external to the organs. Factors affecting the quantitative and qualitative variations in the bacterial content of fresh marine fish are reported by Shewan (32) to depend on the method of capture. Bedford (2) points out that the struggling of line-caught halibut may cause disruptions of the intestinal tract which results in contamination of the visceral cavity. No such disruptions were evident in any of the fish examined in this survey. Very seldom, too, was more than one or two species of bacteria obtained from any tissue examined. While the possibility exists that some contamination from external sources occurred, great care was taken to prevent this happening.

The controversy about whether the internal organs and flesh of fresh, healthy fish are bacteriologically sterile is still not conclusively settled. Bruns (8) and Procter and Nickerson (26) support the view that normal fish tissue is sterile, but Bisset (4) found that inoculum aseptically taken and cultivated from the peritoneal cavities of healthy fish showed the presence of what he considered to be normal, saprophytic water bacteria. Moreover, he was able to demonstrate agglutinins in the serum of fish blood which agglutinated the isolates taken from these fish, while the serum failed to agglutinate bacteria not likely to be found in unpolluted water. He therefore concluded that fish may become parasitized by bacteria commonly found in their environment. In the present investigation, no bacteria were isolated from 32% of the hearts, 28% of the kidneys, and 28% of the livers of the 350 fish examined.

The distribution of the various bacterial genera isolated from the fish in all the areas sampled, the consistency of occurrence of each bacterial genus encountered in each catch of fish, and the frequency with which each bacterial

genus was isolated from all the fish examined are expressed in Table II as the distribution index, consistency index, and frequency index, respectively.

Of the samples of fish taken, only four species of trout were examined in large enough numbers to attempt any comparison of the bacterial flora associated with each species. The results presented in Table III are based on isolations from random samplings of fish taken wherever they could be captured during the period from June, 1958, to December, 1959. These results indicate that, in general, a qualitatively similar bacterial flora is shared by each of the trout species, and bacteria of the genera *Pseudomonas*, *Aeromonas*, *Micrococcus*, and *Lactobacillus* are consistently present in largest numbers. Bacteria of these genera were present in over 10% of each species of fish, while no single

TABLE II

The distribution, consistency, and frequency indices of the bacterial genera isolated from fresh-water fish

Bacterial genera	Distribution index (%, 43 areas)	Consistency index (%, 50 catches)	Frequency index (%, 350 fish)
<i>Pseudomonas</i>	65	60	29
<i>Aeromonas</i>	64	62	24
<i>Micrococcus</i>	65	56	20
<i>Lactobacillus</i>	53	46	19
<i>Escherichia</i>	39	36	13
<i>Brevibacterium</i>	39	36	12
<i>Paracolobactrum</i>	30	26	6
<i>Aerobacter</i>	27	24	6
<i>Proteus</i>	23	20	4
<i>Bacillus</i>	18	16	3
<i>Alcaligenes</i>	16	14	3
<i>Achromobacter</i>	14	12	2
<i>Streptococcus</i>	9	8	1
<i>Flavobacterium</i>	7	6	1

TABLE III

Frequency of isolation (%) of bacterial genera from internal organs of four species of trout

Bacterial genera	Trout species			
	151 brook	79 rainbow	69 brown	37 lake
<i>Pseudomonas</i>	24.5	25.3	21.8	59.5
<i>Aeromonas</i>	22.5	17.7	23.2	37.8
<i>Micrococcus</i>	11.9	31.6	29.0	18.0
<i>Lactobacillus</i>	13.2	11.4	26.0	51.4
<i>Escherichia</i>	11.9	19.0	1.1	16.2
<i>Brevibacterium</i>	7.3	16.4	17.4	18.9
<i>Paracolobactrum</i>	7.3	2.5	2.9	16.2
<i>Aerobacter</i>	3.3	7.6	7.2	5.4
<i>Proteus</i>	6.6	1.3	1.4	5.4
<i>Alcaligenes</i>	3.3	0.0	1.4	16.2
<i>Bacillus</i>	2.7	3.8	2.9	5.4
<i>Achromobacter</i>	3.3	3.8	0.0	0.0
<i>Flavobacterium</i>	1.3	0.0	0.0	8.1
<i>Streptococcus</i>	0.7	0.0	4.3	0.0

genus of the other 10 bacterial genera identified occurred in all fish species at such a rate. However, higher values than 10% are observed in individual fish species for the genera *Escherichia*, *Brevibacterium*, *Paracolobactrum*, and *Alcaligenes*.

It is interesting to note that bacteria of the genus *Pseudomonas* and genus *Aeromonas* predominated in the 44 slime samples taken from the fish, 68% of such slime samples containing species of the genus *Pseudomonas*, and 14% containing species of the genus *Aeromonas*. Bacteria of the genus *Pseudomonas* also predominated in the water samples taken from 11 of the 43 areas from which fish were obtained, 73% of these water samples containing bacteria of this genus.

While there appeared to be a number of bacteria associated with the fresh-water fish which could not be studied by the methods employed, the evidence suggests that members of the genera *Pseudomonas*, *Aeromonas*, *Micrococcus*, and *Lactobacillus* predominate in those internal organs examined of the fish from the areas sampled. Representatives of the following genera were also found to be present in decreasing order of occurrence: *Escherichia*, *Brevibacterium*, *Paracolobactrum*, *Aerobacter*, *Proteus*, *Alcaligenes*, *Achromobacter*, *Streptococcus*, and *Flavobacterium*. A qualitatively similar bacterial flora was obtained from the various species of trout examined.

The results of this survey agree qualitatively with those obtained by Thjøtta and Sømme (41) and Venkataraman and Sreenivasan (43) in that the following genera of bacteria were isolated from fresh-water fish: *Pseudomonas*, *Micrococcus*, *Achromobacter*, *Flavobacterium*, *Escherichia*, *Proteus*, *Paracolobactrum*, *Aerobacter*, *Bacillus*, and *Alcaligenes*. The results differ in that members of the genera *Aeromonas*, *Lactobacillus*, *Brevibacterium*, and *Streptococcus* were isolated in this survey only, while those of the genera *Chromobacterium*, *Salmonella*, and *Shigella* were isolated only by Thjøtta and Sømme, and those of the genera *Bacterium*, *Sarcina*, and *Microbacterium* by Venkataraman and Sreenivasan only. Quantitative differences were obtained in all the bacterial genera isolated in these three investigations. Such qualitative and quantitative differences are to be expected, however, considering the sources from which isolations were made and the different systems of bacterial classification followed.

The bacterial flora of the Ontario fresh-water fish examined was similar to that reported from marine fish in that species of *Pseudomonas* and *Micrococcus* were encountered frequently, but differed in that species of *Achromobacter* and *Flavobacterium* occurred infrequently.

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THE FORMATION OF CELLULOSE MICROFIBRILS BY ACETOBACTER XYLINUM IN AGAR SURFACES¹

B. MILLMAN² AND J. ROSS COLVIN

Abstract

The formation of extracellular cellulose microfibrils by *Acetobacter xylinum* on agar surfaces is remote from the cell membrane and does not involve an intermediate, amorphous high polymer, in agreement with conclusions from studies of liquid suspensions. Growth of individual microfibrils is at the tip(s) only and the rate of extension (0.2μ per bacterial cell per minute at 34°C) is comparable with that in liquid medium. The rate of nucleation of new microfibrils is about 40 per 10^8 bacteria per minute at 34°C . Both rates are constant after an induction period of about 30 seconds. Newly nucleated microfibrils could be identified unequivocally down to a length of 0.5μ . A characteristic feature of growth of cellulose on agar surfaces is the formation of bundles of microfibrils with their axes roughly parallel. The results suggest that the rate-limiting step in the formation of these microfibrils has an activation energy of about 15 kc.

Introduction

The mechanism of formation of cellulose microfibrils by *Acetobacter xylinum* has been studied intensively because it may offer insight into cellulose synthesis in higher plants, but many aspects are still obscure. Recent evidence indicates that the microfibrils are formed extracellularly, remote from the cell membrane (4) and without an intermediate high polymer (3). However, these conclusions were based on experiments with cells in liquid suspensions where fragile associations between microfibrils and the cell surface might easily be broken. The study has therefore been extended to growth of bacterial cellulose microfibrils on moist agar surfaces, where both microfibrils and cells are held stationary during specimen preparation. These conditions decrease the possibility of breaking any delicate microfibril-surface attachments which may exist, and are also favorable for study of short microfibrils and their rates of growth.

Materials and Methods

Suspensions of washed, cellulose-free cells of *A. xylinum* were prepared and stored as described previously (2). Immediately before use, the cells were filtered through several layers of cheesecloth over fiber-glass wool and then diluted 1:5 with buffer, 0.01 *M* in phosphate, pH 6.0.

Smears of bacterial cells on agar surfaces were prepared as follows. Bacto-agar (1.5 g) and glucose (1.9 g) were dissolved in 100 ml of boiling water, poured into Petri dishes, and autoclaved. The cooled agar plates were inoculated by drawing a glass rod, which had been dipped in the diluted, filtered cell suspension, lightly across the surface. Visible water from the cell smear was absorbed in 5–10 seconds. Subsequently, no motion of individual cells or colonies on the agar surface was detectable by the optical microscope. Incu-

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bation temperature was about 30° C for qualitative studies and 34° C for quantitative estimates. Metabolism of the cells in the smears was stopped by placing the agar plate in a formaldehyde gas chamber.

Collodion pseudoreplicas of the bacteria on the agar were prepared by spreading a drop of collodion solution on the surface, allowing the film to dry, and then floating it off on water. No disturbance of the bacterial colonies by the collodion solution was apparent. After they were washed by being floated on distilled water, the pseudoreplicas were placed on copper grids, dried, shadowed with Pd-Au (60-40) at an angle of 15° and examined in the electron microscope.

For quantitative studies, pseudoreplicas were obtained as above, except that the incubation temperature was 34° C and the time of incubation was closely controlled. A representative grid square was selected in the pseudoreplica from each incubation interval and photographed completely at 1300 magnification. The number of microfibrils of a given length in the grid square was counted (class interval 0.4 μ), together with the number of bacterial cells in a defined central portion of the grid square. This number of bacteria, multiplied by the ratio of the area of the grid square to the area of the defined central portion, gave an estimate of the total number of bacteria in the grid square. These data allowed estimation of mean microfibril length, numbers of microfibrils of a given length, and total microfibril length per bacterial cell as a function of time of incubation.

Results

Photographs of pseudoreplicas, prepared from agar surfaces which carried metabolizing *A. xylinum* cells, confirmed the previous conclusion that growth of the microfibrils takes place remote from the cell wall (Figs. 1 and 2). If a consistent association between microfibrils and surfaces of cells existed, it should have been demonstrable with the technique used but none was observed. The pseudoreplicas also showed no evidence for an extensive, amorphous, intermediate high polymer, in agreement with the results from liquid cultures (3). In some experiments, washing of the pseudoreplicas was omitted in order to minimize loss of soluble substances but these films also gave no evidence of such a polymer.

As in liquid suspensions, individual microfibrils on agar do not increase in diameter with time but grow in length only (Figs. 1 and 2). This means that the microfibrils must grow at the tip(s), which were tapered (Fig. 2). The intertwining of separate microfibrils, to form bundles during linear growth, which has been reported in liquid suspensions (3) is also present on agar surfaces (Figs. 1 and 2). No distinction between the (two) ends of a microfibril could be detected.

The results of a quantitative study of the rates of formation and of linear growth of cellulose microfibrils on agar surfaces are shown in Figs. 3 to 6. Figure 3 shows clearly that there is an induction period of approximately 30 seconds in the rate of microfibril growth at 34° C on agar, after which the rate is constant at about 0.2 μ per bacterial cell per minute. As shown in Fig. 4 the mean length of microfibrils increases steadily from the time they first become detectable and, in contrast to results in liquid culture, the standard deviation



FIG. 1. Bacterial cellulose microfibrils grown on an agar surface, 30 minutes' incubation time.

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PLATE II

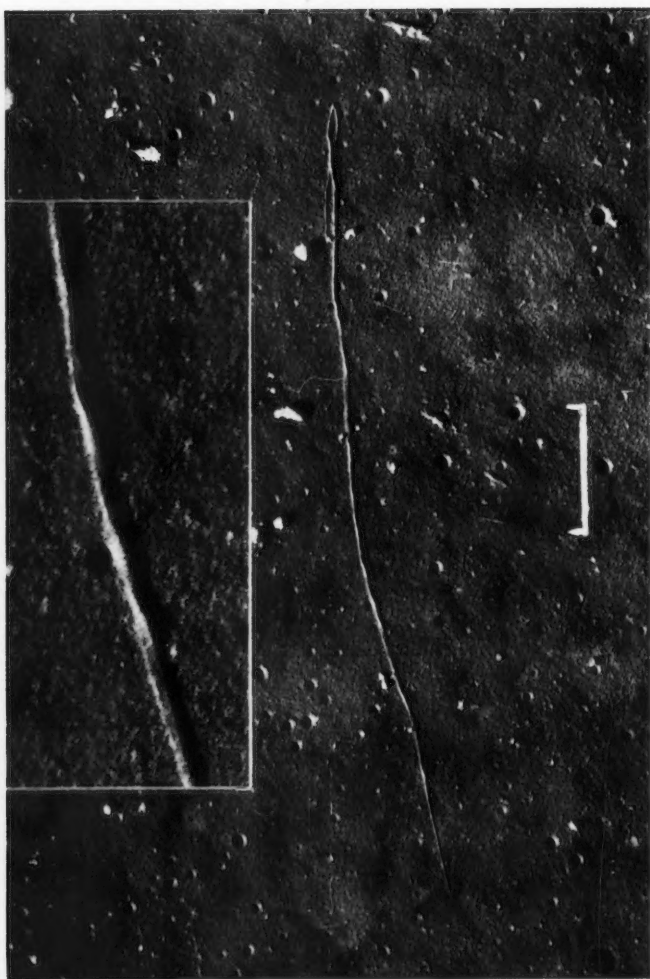


FIG. 2. A pair of microfibrils grown on an agar surface. Note tapering of both tips. Insert: note typical twist. Millman and Colvin—Can. J. Microbiol.

of the mean remains roughly the same. In Fig. 5 is shown a typical distribution of microfibril lengths on an agar surface, for 90 seconds' incubation. The skew distribution is due, at least in part, to the nucleation of new short microfibrils as incubation proceeds. The rate of initiation of new microfibrils per thousand bacteria on an agar surface is shown in Fig. 6 and is roughly 40 per minute at 34° C.

The pseudoreplica technique facilitated identification and study of short, presumably newly nucleated microfibrils down to a length of about 0.5 μ (Figs. 7 and 8). Smaller linear aggregates could be observed but unequivocal

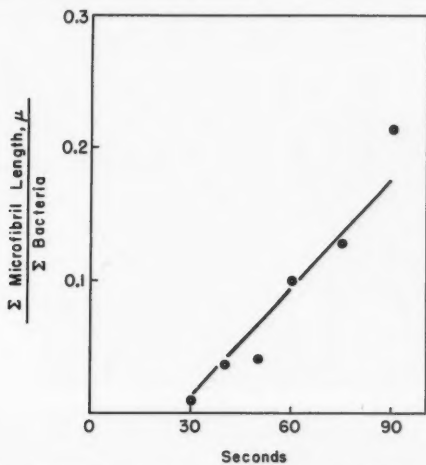


FIG. 3. Total microfibril length per bacterial cell as a function of time.

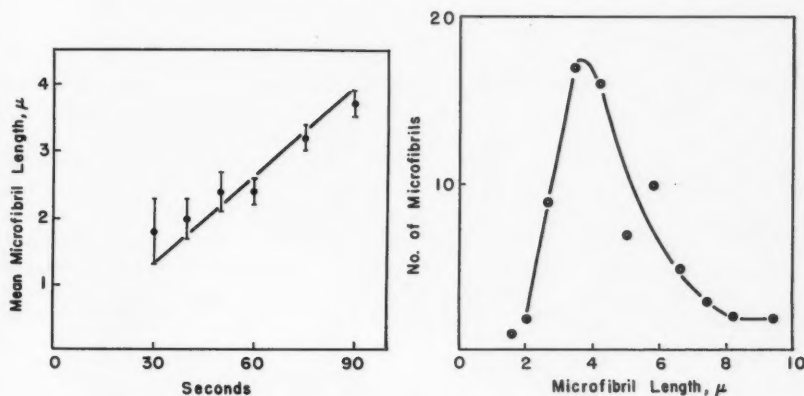


FIG. 4. Mean microfibril length as a function of time.

FIG. 5. A typical distribution of microfibril lengths, after 90 seconds' incubation.

identification of these as microfibril initials was not possible because some particulate debris is always introduced from the cell suspension. The short, newly nucleated microfibrils were of the same diameter as older, longer threads, with no obvious differences between the two classes other than length.

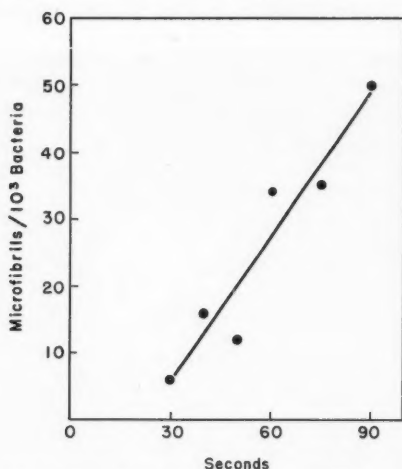


FIG. 6. The rate of formation of microfibrils per thousand bacteria on agar surfaces at 34° C.

A striking feature of growth of microfibrils on agar surfaces is shown in Fig. 9. Short microfibrils were often observed clumped together in sheaves or bundles, with the axes of the microfibrils roughly parallel. It was not possible to say whether short, separately formed, microfibrils associated with each other to form bundles by van der Waals forces, or the nuclei of nascent microfibrils were laid down parallel to and in close association with older threads.

Discussion

The foregoing results from agar surfaces corroborate previous conclusions that the extracellular bacterial cellulose microfibril increases in size by tip growth remote from the cell surface and without an amorphous, high polymeric intermediate (2). They also show that when washed, intact *A. xylinum* cells are introduced into buffered glucose, an induction period of about 30 seconds must elapse before a detectable steady rate of bacterial cellulose synthesis is established. This induction period may be required to build up a necessary steady-state concentration of precursor in the external medium, to secrete sufficient extracellular enzyme (1) into the medium, or to establish a mechanism for nucleation of new microfibrils, but no definitive information on any of these alternatives is available yet.

FIG. 7. Two short (~ 0.5 and 1.0μ), presumably newly nucleated microfibrils on an agar surface, 1 minute incubation time. Note typical twisted bundle of microfibrils below.

FIG. 8. A longer newly nucleated microfibril (~ 1.5 – 2.0μ) grown on an agar surface, 1 minute incubation time.

PLATE III

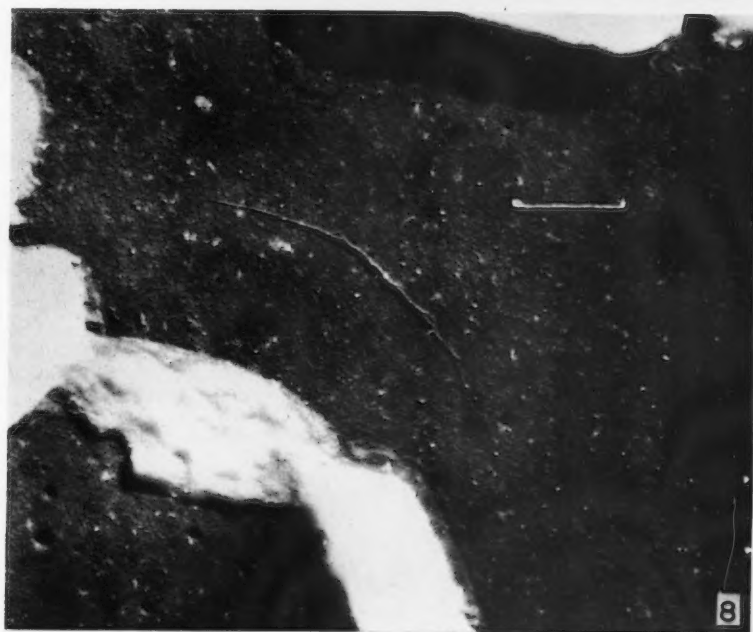




FIG. 9. Bundle of short microfibrils grown on agar, 120 minutes' incubation time.
Millman and Colvin—Can. J. Microbiol.

The steady increase in mean microfibril length coupled with a stationary standard deviation also suggests that the allocation of glucose residues to the ends of individual microfibrils is essentially a random process. No microfibrils have a preferred position or advantage, such as the continued adsorption of enzyme to particular microfibril tips. The moderately skewed distribution of microfibril lengths, due to continuous introduction of new short microfibrils, is also consistent with the concept that once an activated glucose residue is secreted into the external medium, there are roughly equal probabilities that it will be attached to any of the individual microfibrils in the vicinity or be used to nucleate new fibrils.

The above rates of nucleation of new microfibrils and of linear growth in water films on agar at 34° are approximately double the rates for the same processes in liquid suspension at 25° C. This approximate doubling of both rates for a temperature increase of 9° suggests that the rate-limiting step in the synthesis of cellulose in colonies of *A. xylinum*, either in liquid or on agar, involves breaking a covalent bond with an activation energy of about 15 kc. If this is correct, the rate-limiting reaction must precede the nucleation step in the over-all process.

The close intertwining of individual growing bacterial cellulose microfibrils in liquid suspension or on agar surfaces, and particularly the formation of sheaves of short microfibrils on agar surfaces are probably a reflection of interactions between single separate microfibrils at short distances. Similar interactions are possibly of importance in the orientation of microfibrils within plant cell walls.

Acknowledgments

The careful technical assistance of Mr. L. Sowden is gratefully acknowledged.

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THE BREAKDOWN OF RIBONUCLEOSIDES IN EXTRACTS OF THE PHYTOPATHOGENIC ORGANISM *XANTHOMONAS PHASEOLI*¹

R. M. HOCHSTER AND C. G. NOZZOLILLO²

Abstract

Extracts of *Xanthomonas phaseoli* (XP8) degrade certain ribonucleosides readily by hydrolytic cleavage of the ribose moiety. Thus, uridine is converted to uracil and ribose, and cytidine to cytosine and ribose, with little deamination to uracil and ammonia taking place. Commercial adenosine is deaminated to inosine and ammonia without further significant cleavage. The data presented permit a choice to be made between the two theoretically possible pathways of guanosine degradation. With chromatographic evidence for xanthine but not for xanthosine and with spectrophotometric evidence for the intermediate formation of guanine, the mechanism of guanosine breakdown is described as being the result of hydrolytic cleavage to guanine and ribose followed by deamination to xanthine and ammonia.

Introduction

Kalckar (10) first described the hydrolysis of inosine in several species of *Lactobacillus*, while the hydrolytic cleavage of uridine was reported in bakers' yeast by Carter (2). A wide distribution of an alternate phosphorylative mechanism has, however, been recognized for some time (3, 12). In fact, Heppel and Hilmo (6) achieved a complete separation of phosphorylative from hydrolytic enzymes catalyzing the cleavage of purine nucleosides in yeast. The existence of separate hydrolytic enzymes specific for purine and for pyrimidine nucleosides was shown by Lampen and Wang (13) in *Lactobacillus pentosus*. Tarr (23) showed that fish muscle contains a riboside hydrolase which hydrolyzes purine ribosides and cytidine and another which is specific for the hydrolysis of inosine. Heat-stable enzymes capable of hydrolyzing various nucleosides have also been reported to be present in spores of *Bacillus cereus* (14, 15, 18). Takagi and Horecker (22) have shown that the riboside hydrolase of *Lactobacillus delbrueckii* (21) catalyzes the hydrolytic cleavage of a large number of natural and synthetic ribosides, the effect being specific for the β -ribofuranoside linkage. On the other hand, deaminases have been described for cytosine (3, 5, 24), cytidine (20, 24), adenosine (8, 18), and guanosine (19) in different biological tissues and in microorganisms.

A complete study on the mechanism of breakdown of adenosine phosphates to hypoxanthine by extracts of the plant pathogenic organism *Xanthomonas phaseoli* (XP8) has been published from this laboratory (8). Subsequent work on the mechanism of deoxyribose formation in this organism, in which a variety of nucleosides were intended to serve as substrates, led to the realization that nucleosides themselves break down readily in the presence of such cell-free extracts. It was found necessary to obtain such information in

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quantitative form in order to render possible a clear-cut interpretation of subsequent enzymatic studies being carried out in our laboratory with extracts of *X. phaseoli*. The results of this work are presented in this report.

Materials and Methods

Materials

The following substances used in this investigation were commercial preparations: uridine, guanosine, cytidine, adenosine, inosine (Pabst Brewing Co.); tris(hydroxymethyl)aminomethane ("tris"), hypoxanthine, adenine, cytosine, guanine, uracil, xanthine (Sigma Chemical Co.); D-ribose (Pfanzstiel Laboratories) and xanthosine, D-ribose-5-phosphate (Nutritional Biochemicals Corp.). D-Ribose-1-phosphate was prepared according to Kalckar (9) and isolated as the barium salt.

Methods

(a) *Preparation of extracts*.—Cells of *X. phaseoli* (XP8) were grown in a medium consisting of 1% yeast extract and 2% glucose for a period of 16 hours in Fernbach flasks on a rotary shaker at 25–27° C. The resulting cells were harvested and washed three times with 0.9% NaCl and then suspended in two volumes of 0.02 M tris buffer (pH 7.3). This suspension was treated in a Raytheon, 10-kc sonic oscillator for 5 minutes at a power output of 1.0 amp followed by centrifugation at $13000 \times g$ at 0° C for 10 minutes. A molar solution of $MnCl_2$ was then added in the proportion of 0.04 ml per ml of extract obtained in order to remove nucleoprotein. After intimate mixing, the preparation was allowed to stand at room temperature for 10 minutes, followed by centrifugation as above. The resultant supernatant suspension was then dialyzed for 1 hour against running tap water (5–8° C) and for an additional hour against distilled water at 0–4° C. This preparation had an average protein content of 36–38 mg/ml (25).

(b) *Analysis for ammonia*.—This was performed by a modification of the microdiffusion method of Conway (4), as follows. Three-milliliter circular pH-meter cups were each covered with a micro cover glass, and tight closure was achieved by means of a ring of stopcock grease applied to the underside of the cover glass. The sample cup contained 0.1 ml of material to be assayed, and 0.01 ml *N* HCl was added to the underside of the cover slip in the form of a hanging drop. At zero time, 0.1 ml 50% KOH was added to the contents of the cup and the cover slip was immediately pressed down to seal. The contents of the cup were mixed by gentle swirling and allowed to stand for 30 minutes. At that time, the cover slip was removed and the hanging drop of HCl was washed into the cup with 2.8–2.9 ml of water. Nessler's reagent (0.1–0.2 ml) was added immediately and the resultant color read at 425 $m\mu$ in a Beckman DU spectrophotometer. Under these conditions, the test gave a linear response between 0–1.0 μ mole of ammonia per ml.

(c) *Analysis for ribose*.—Aliquots of reaction mixtures were placed on paper chromatograms and developed and sprayed as previously reported (7) after suitable treatment to remove protein. Corroborative evidence for the identity of the pentose as ribose was obtained by paper electrophoresis (8). For quantitative determinations, the ribose-containing areas were cut out from the

chromatograms and eluted with 2.5 ml of water. An aliquot was then assayed by the method of Mejbaum (17), using a 40-minute boiling time.

(d) *Chromatographic separation of reaction products.*—Nucleoside breakdown was studied by incubation of nucleosides with extracts in tris buffer (pH 7.3) at 30° C. Enzymatic reactions were stopped by boiling the mixture for 10 minutes followed by chilling and centrifugation. Aliquots of such reaction mixtures (0.005 ml) were transferred to Whatman No. 1 filter paper along with known reference compounds and were subjected to descending chromatography in one of two solvent systems. Reaction mixtures in which adenosine, cytidine, or uridine were used as substrates were run in isobutyric acid: concentrated NH_4OH :water in the proportions 66:1:33^a while guanosine reaction mixtures were run in ethanol: 1.0 *M* ammonium acetate (pH 7.5) in the proportions 7:3.^a Compounds were located on the dried papers by exposure to a Mineralight (Model SL 2537) in a dark room. (For R_f values, see Table I below.) Rectangles were cut from the papers (2.5 cm wide) and long enough to include all of the respective ultraviolet-absorbing areas. Corresponding

TABLE I
Physical constants used in chromatography and spectrophotometry

Compound	R_f		Spectrophotometric analysis		
	System I	System II	λ_{max}	$E_{\text{max}} \times 10^3$	pH
Adenosine	0.77	0.63	259	15.4*	7.0
			259.5	14.9	6.4
Inosine	0.47	0.60	248.5	12.2*	6.0
Cytidine	0.59	0.65	280	13.0*	2.0
			271	8.9	7.0
Uridine	0.43	0.73	262	10.0*	7.0
Guanosine	0.47	0.56	252	13.7*	7.0
			252.5	13.65	6.0
			260	11.30	N NaOH†
Xanthosine	0.40	0.42	278	8.9	7.0
			276	9.3	N NaOH†
Adenine	0.85	0.65	260.5	13.35	7.0
Hypoxanthine	0.59	0.61	249.5	10.7	6.0
Cytosine	0.74	—	267	6.13	7.2
Uracil	0.59	—	259.5	8.2	7.2
Guanine	—	0.49	275.5	8.15	7.0
			274	9.9	N NaOH†
Xanthine	0.50	0.48	267	10.25	2.8
			284	9.4	N NaOH†
Ribose	0.41	0.73	—	—	—

*From Pabst Circular OR-15, 1959. E_{max} values not marked by asterisk taken from the *Nucleic Acids*, Vol. 1, by E. Chargaff and J.N. Davidson, Academic Press, 1955.

†Not buffered.

^aSystems I and II respectively, published by Pabst Brewing Co., in their circular OR-15, p. 4 (1959).

blanks were always cut from the control reaction chromatograms. The small paper strips thus obtained were covered in small tubes with 3.0 ml of 0.02 *M* phosphate buffer (pH 7.0) and allowed to stand for 2 hours at room temperature or they were placed in a boiling water bath for 15 minutes (e.g. in the case of the guanosine experiments) where, occasionally, *N* NaOH was also used as an eluting medium (depending upon the pH at which the analyses were to be carried out). The resulting solutions were analyzed spectrophotometrically using both a Beckman DU spectrophotometer (for routine determinations of optical density) and a Beckman DK-1 ratio recording spectrophotometer (for the tracing of complete absorption spectra). The physical constants used are given in Table I as are the R_f values obtained on the chromatograms. In cases where complete chromatographic separations were not achieved (as with guanosine and xanthine and with cytidine and uracil) the over-all spot was cut out and eluted and the eluate analyzed by application of the series of general equations given by Loring (16) for the optical densities at two wave lengths.

It should be emphasized here that all values given in the experimental details below (with the exception of those for ammonia) are the result of reisolation of each substance for each time-period sample taken, followed by analysis. Thus, possible interference in the analytical work by other substances present in the bacterial extracts has been reduced to a minimum, although it is recognized that the data are subject to the limitations of reisolation from paper chromatograms.

Experimental and Results

Uridine Degradation

Uridine was degraded to uracil and ribose (Fig. 1) at a steady but slow rate. It required approximately 45 minutes for half the substrate to be converted. Whereas the conversion to uracil was stoichiometric, ribose recovery fell off considerably after 30 minutes' incubation; the reason for this is, at

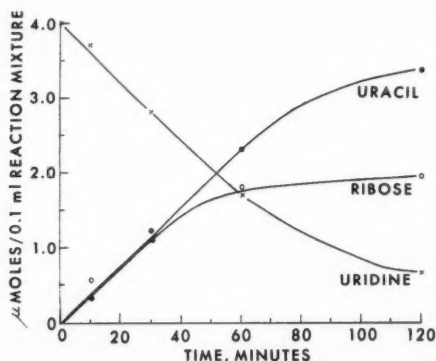


FIG. 1. The breakdown of uridine by an extract of *X. phaseoli*. Contents of reaction mixture: uridine, 120 μ moles; tris buffer (pH 7.3), 175 μ moles; $MnCl_2$ -treated extract, 1.0 ml. Temperature of incubation: 30° C, total volume: 3 ml.

present, unknown. The functioning of a possible transribosidase (11) must be ruled out since our chromatograms did not reveal the formation of any new ribose-containing nucleoside. Furthermore, there was no evidence for ribose phosphate formation or further degradation of uracil under these conditions.

Cytidine Degradation

The rate of cytidine conversion (Fig. 2) was similar to that reported for uridine (Fig. 1). Stoichiometric conversion took place, while the rate of ribose release was parallel to cytosine formation during the first hour. It was realized that, theoretically at least, cytosine could give rise to uracil by deamination. Experimentally, 3.16 μ moles of cytidine were converted to 2.94 μ moles of cytosine in 2 hours with the concomitant formation of 0.30 μ mole of uracil

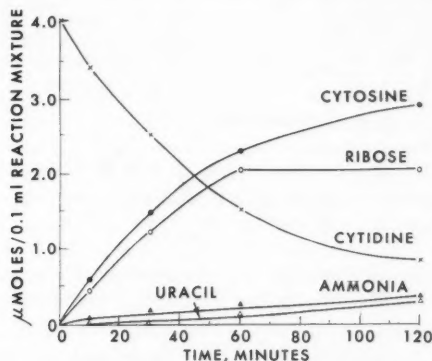


FIG. 2. The breakdown of cytidine by an extract of *X. phaseoli*. Contents of reaction mixture: as in Fig. 1, except that cytidine was used as substrate.

and a measured quantity of 0.36 μ mole of ammonia on the basis of 0.1 ml of reaction mixture. Thus the deamination reaction is either extremely weak and of little significance, or perhaps quite different experimental conditions are required to demonstrate it. In view of the fact that the conditions chosen for this work were such that the results could be used along with those previously recorded (8) to achieve a better understanding of over-all nucleotide and nucleoside metabolism in *X. phaseoli*, other reaction conditions were not tried. We could not find any evidence for the theoretically possible formation of ribose phosphate.

Adenosine Degradation

The enzymatic deamination of adenosine to inosine and its subsequent deribosylation to hypoxanthine have been described previously (8). In the latter report the first reaction was studied manometrically while the actual accumulation of hypoxanthine was demonstrated in experiments in which adenylic acid served as the substrate. In view of the report of Koch (11) that the inosine-splitting reaction was inhibited by adenine and since we found that commercial adenosine contained adenine as an impurity, degra-

dation experiments were carried out with commercial adenosine as substrate. As shown in Fig. 3, adenosine is almost totally converted to inosine, confirming our previous observation by the manometric method, but when adenosine was the substrate, the deribosylation reaction to hypoxanthine was strongly inhibited. Relatively little hypoxanthine or ribose accumulated under these conditions thus confirming, indirectly, the results of Koch in extracts of *E. coli*. Again, no evidence could be obtained for the possible formation of ribose phosphate in these incubations.

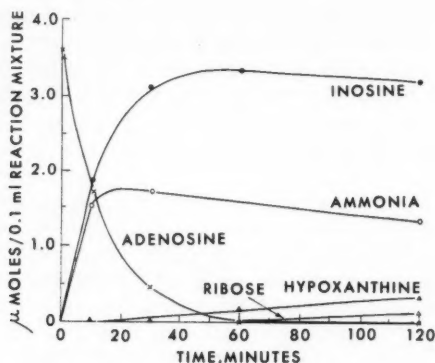
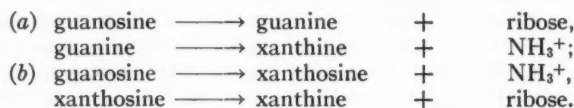


FIG. 3. The breakdown of adenosine by an extract of *X. phaseoli*. Contents of reaction mixture: as in Fig. 1, except that adenosine was used as substrate.

Guanosine Degradation

Two theoretically possible pathways can be formulated for the degradation of guanosine both of which have the same end result. They differ only in that the first intermediate is different in each case, depending on whether deribosylation or deamination is the first reaction. Thus:



It is immediately apparent (see Fig. 4) that guanosine disappears at a much faster rate than do any of the other ribonucleosides used in this study. Ribose and ammonia are found quite early and, in a fast-reaction sequence as is exhibited here, it is difficult to render any judgment about whether ribose or ammonia is liberated first. The accumulating ultraviolet-absorbing material was analyzed chromatographically and spectrophotometrically (for details, see Materials and Methods) and was found to correspond to xanthine rather than to xanthosine. This finding then led to a search for the missing guanine, indicated by a dotted line in Fig. 4 (quantities calculated by difference between guanosine disappearance and xanthine formation). Since it was difficult to obtain clear-cut separation of xanthine and guanine on chromatograms, it was decided to obtain complete spectra of the ultraviolet-absorbing

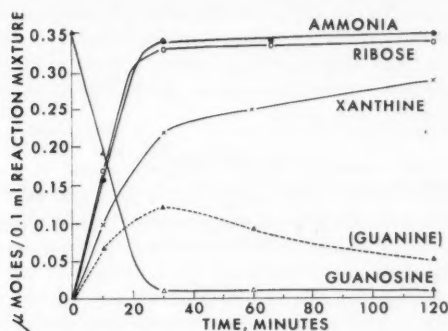


FIG. 4. The breakdown of guanosine by an extract of *X. phaseoli*. Contents of reaction mixture: as in Fig. 1, except that guanosine (10.5 μ moles) was used as substrate. The low solubility of guanosine dictated its use in lower concentrations.

components of aliquots which had previously been obtained by elution from sections of such chromatograms (as detailed in Materials and Methods). The result of one such determination (10-minute reaction sample) is given in Fig. 5. At pH 7.0 (1) xanthine was found to have a single peak at 268 $m\mu$ and guanine gave a double-peak curve with maxima at 245.4 and at 274.3 $m\mu$. The experimental sample (curve 3) gave a double-peak curve with maxima at 246 and 270 $m\mu$. This is interpreted as representing guanine formation under conditions where the xanthine concentration is already quite considerable (see Fig. 4), hence the displacement of the second peak as influenced by the relatively high xanthine concentration at this particular point.

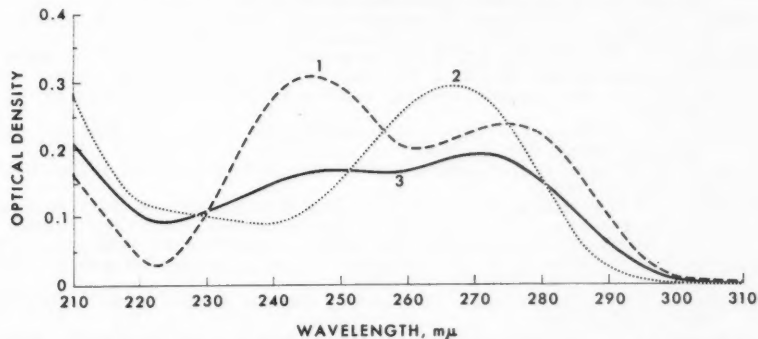


FIG. 5. Spectrum (pH 7.0) of the ultraviolet-absorbing components of a 10-minute reaction sample following incubation of guanosine with extract (conditions as in Figs. 1, 4). Curve 1 = guanine (control), curve 2 = xanthine (control), curve 3 = experimental sample.

Possible Enzymatic Hydrolysis of Ribose Phosphates

In view of the fact that no evidence was obtained in any of this work for the existence of a phosphorolytic cleavage of the nucleosides studied, it was

of importance to establish whether or not the extracts used would dephosphorylate ribose-1-phosphate or ribose-5-phosphate. It was found that no inorganic phosphate was released from either ester when incubated up to 60 minutes with extracts under the same conditions that were used for the experiments reported in this paper.

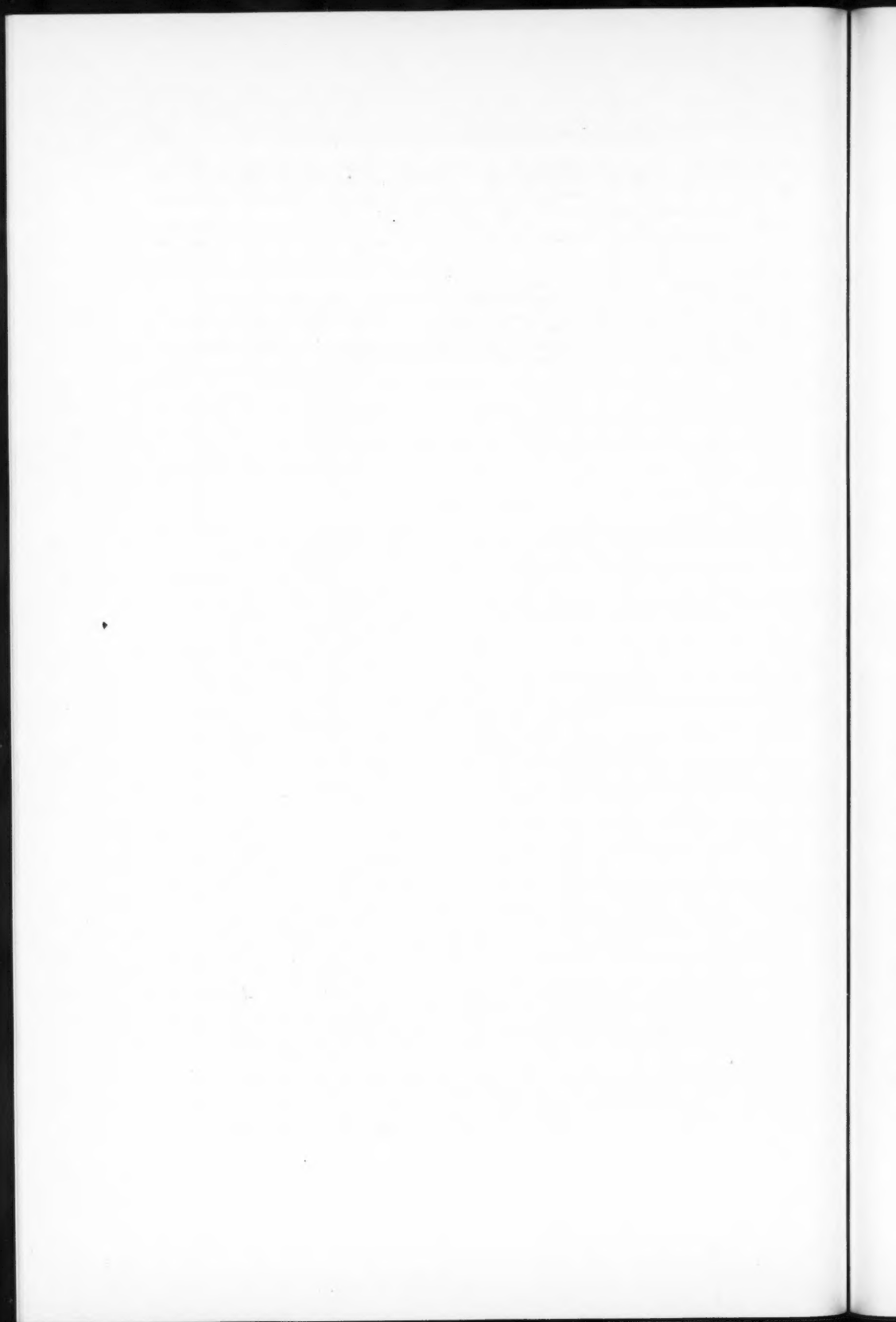
Discussion

The foregoing data show that extracts of *X. phaseoli* readily degrade ribonucleosides. The evidence presented supports the contention that the splitting reactions observed are of the hydrolytic type and are not phosphorylytic. The extracts used were dialyzed and inorganic phosphate was not supplied. Ribose phosphates did not appear on any of the many chromatograms that were examined and ribose-1-phosphate and ribose-5-phosphate were not hydrolyzed by these preparations. The data further support mechanism *a* for the degradation of guanosine.

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THE EFFECT OF SALINITY ON GROWTH AND CHLOROPHYLL CONTENT IN REPRESENTATIVE CLASSES OF UNICELLULAR MARINE ALGAE¹

JACK McLACHLAN²

Abstract

Growth and relative chlorophyll content per cell of *Platymonas* sp., *Syracosphaera carterae*, *Monochrysis lutheri*, *Olisthodiscus* sp., *Thalassiosira decipiens*, *Cyclotella* sp., *Cryptomonas* sp., *Amphidinium carteri*, and *Porphyridium* sp. were determined at various salinities from 2.5 to 35 parts per thousand in an enriched sea-water medium.

All of these organisms grew well in a wide range of salinities, and when growth was completely suppressed it was at the lower salinities. *Thalassiosira*, *Cyclotella*, *Syracosphaera*, *Cryptomonas*, and *Monochrysis* did not show a definite salinity optimum for growth, and the chlorophyll content per cell was approximately the same at all salinities. *Olisthodiscus* and *Amphidinium* showed a salinity optimum for growth, and the chlorophyll content per cell was also higher at the optimum salinity for growth. Growth of *Platymonas* and *Porphyridium* was little influenced by changes in salinity, but the chlorophyll content per cell was affected by changes in salinity.

Introduction

Salinity is considered an important ecological variable in the marine environment, particularly in inshore areas (11, 14). Completely euryhaline organisms (i.e. organisms which are capable of moving from fresh water to full strength sea water) are relatively uncommon (10). Blinks (1) mentions observations of marine algae growing on the hulls of ships which move daily from fresh water to salt water. However, most of the larger marine algal species cannot tolerate dilutions below $0.5\times$ or concentrations of more than $1.5\times$ sea water. Laboratory studies with unicellular marine algae (cf. 2, 3, 4, 12, 13) have shown that most species in culture are tolerant of a wide range of salinities, although generally these organisms show a preference for lower salinities.

In a previous report (7), *Dunaliella* was shown to have a broad salinity tolerance as determined by cell concentrations. However, an empirical estimation of the chlorophyll content indicated that a narrower range of salinities was necessary for maximum synthesis of this pigment. Also, Kim (5) working with higher plants, showed that, in general, there was an inverse relationship between the chlorophyll content and the salt concentration of the culture solutions. In studies of fresh-water algae, pigment changes have been noted with variations in the nutrients of the medium (cf. 8, 9). The purpose of the present study was to ascertain if, in unicellular marine algae, a general relationship exists between the relative amount of chlorophyll per cell and growth at various salinities.

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Contribution No. 1150 from the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, U.S.A.

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Materials and Methods

Nine different cultures of unicellular marine algae representative of various algal groups were used in this study. A listing of these cultures together with information on the original isolation is presented in Table I. With the exception of *Porphyridium* sp., all cultures were considered axenic (R. Guillard, personal communication).

TABLE I

Cultures of algae used.* Information regarding original isolation (i.e. person, place, and time) is given when known. The initial concentration of cells in the inoculated cultures (N_0) and the duration of the incubation period are also presented

Organism	Isolation	Initial no. cells/ml $\times 10^4$	Days of growth
Chlorophyte flagellate <i>Platymonas</i> sp.	R. Guillard, Great Pond, Falmouth, Mass., 1954	4.20	7
Chrysophyte flagellates <i>Syracosphaera carterae</i>	I. Pintner, Woods Hole, Mass., 1958	4.18	6
Braarud & Fagerland	M. Droop	4.50	8
<i>Monochrysis lutheri</i> Droop	R. Guillard, Milford, Conn., 1957	0.94	9
<i>Olisthodiscus</i> sp.			
Concentric diatoms <i>Thalassiosira decipiens</i> (Grunow) Jørgensen	R. Guillard, Milford, Conn., 1957	0.035	15
<i>Cyclotella</i> sp.	R. Guillard, West Tisbury, Great Pond, Martha's Vineyard, Mass., 1956	0.58	8
Cryptomonad <i>Cryptomonas</i> sp.	R. Guillard, Milford, Conn., 1957	4.22	6
Dinoflagellate <i>Amphidinium carteri</i> Hulburt	R. Guillard, Great Pond, Falmouth, Mass., 1954	0.30	9
Rhodophyte <i>Porphyridium</i> sp.	R. Lewin	3.60	8

*All cultures were obtained from Dr. R. R. L. Guillard, Woods Hole Oceanographic Institution.

The cultures were grown in an enriched sea-water medium. The sea water was collected from the Sargasso Sea,³ and was passed through a membrane filter (Millipore AA). Glass-distilled water was used to dilute the sea water to the desired salinities varying from 2.5 to 35‰ (parts per thousand). The following enrichments in mmoles/liter were added to the sea-water base: potassium nitrate 1.00, potassium monohydrogen phosphate 0.10, sodium silicate 0.10, disodium ethylenediaminetetraacetate 0.03, tris(hydroxymethyl)-aminomethane 5.00, and micronutrients at the concentration previously used (7). As some of the organisms selected are known to be auxotrophs (11), the following vitamins also were added: thiamine:hydrochloride 0.2 mg/liter, biotin 1.0 μ g/liter, and B₁₂ 1.0 μ g/liter.

*R/V Crawford Cruise 27. Station 553 (May 1959).

The algae were cultured in 125-ml Erlenmeyer flasks containing 35 ml of medium. The cultures were incubated at about 18° C under approximately 3500 meter-candles illumination provided by cool-white fluorescent lights. It was necessary, however, to grow *Porphyridium* sp. in dim, indirect illumination. Growth was estimated by cell counts using a Levy hemocytometer counting chamber, and the results are expressed as $\log_2 N_t/N_0$, which gives the number of times the population has doubled (7). The length of the incubation period and the initial concentration of cells (N_0) in the inoculated cultures are also given in Table I. The concentration of chlorophyll *a* was estimated by the non-extractive method of Yentsch (15), but expressed in arbitrary units giving relative values for each organism. These values should not be compared, since the absolute amounts were quite different for each organism. Measurements were made with a model DU Beckman spectrophotometer at 670 m μ . Some interference with other chlorophylls may be expected at this wave length but probably does not exceed 5% of chlorophyll *a*.

Results

Monochrysis lutheri, *Syracosphaera carterae*, *Cyclotella* sp., *Thalassiosira decipiens*, and *Cryptomonas* sp. did not show a distinct salinity optimum with regard to growth, nor was there a significant difference in the concentration of chlorophyll per cell at the various salinities.

Growth of *Monochrysis* at 2.5‰ was not as dense as at the higher salinities, and the chlorophyll concentration per cell was also low at this salinity. *Syracosphaera* failed to grow at 2.5‰, but growth and chlorophyll concentration per cell at the other salinities were essentially the same. Growth and the chlorophyll concentration of *Cyclotella* were approximately the same from 2.5 to 35‰. *Thalassiosira* did not grow at salinities below 15‰, but growth and the chlorophyll concentration per cell were essentially the same at the higher salinities. *Cryptomonas* grew about equally well at all salinities, and there was no significant change in the concentration of chlorophyll per cell throughout the salinity range.

Growth of *Olisthodiscus* was completely suppressed at 2.5 and 5.0‰ (Fig. 1a). Maximum growth occurred at 15‰, and from 20 to 35‰, growth was essentially the same. A maximum concentration of chlorophyll per cell was also found at 15‰. Therefore, both the cell concentration and chlorophyll per cell content indicate an optimum salinity of 15‰ for this organism.

Maximum growth and chlorophyll concentration per cell occurred at 25‰ for *Amphidinium* (Fig. 1b). There was no growth at salinities from 2.5 to 10‰. The chlorophyll maximum at 25‰, though, was not as well defined in this alga as in *Olisthodiscus*.

Growth of *Porphyridium* sp. occurred at all salinities from 2.5 to 35‰ (Fig. 2a). Growth at 2.5‰ was slightly depressed, but from 5 to 35‰ growth was essentially the same. The concentration of chlorophyll per cell showed a maximum at 25‰.

Platymonas sp. grew at salinities from 2.5 to 35‰; growth from 15 to 35‰ was approximately the same (Fig. 2b). Growth at 2.5‰ was very much reduced, and increased with increasing salinity to 15‰. Maximum chlorophyll

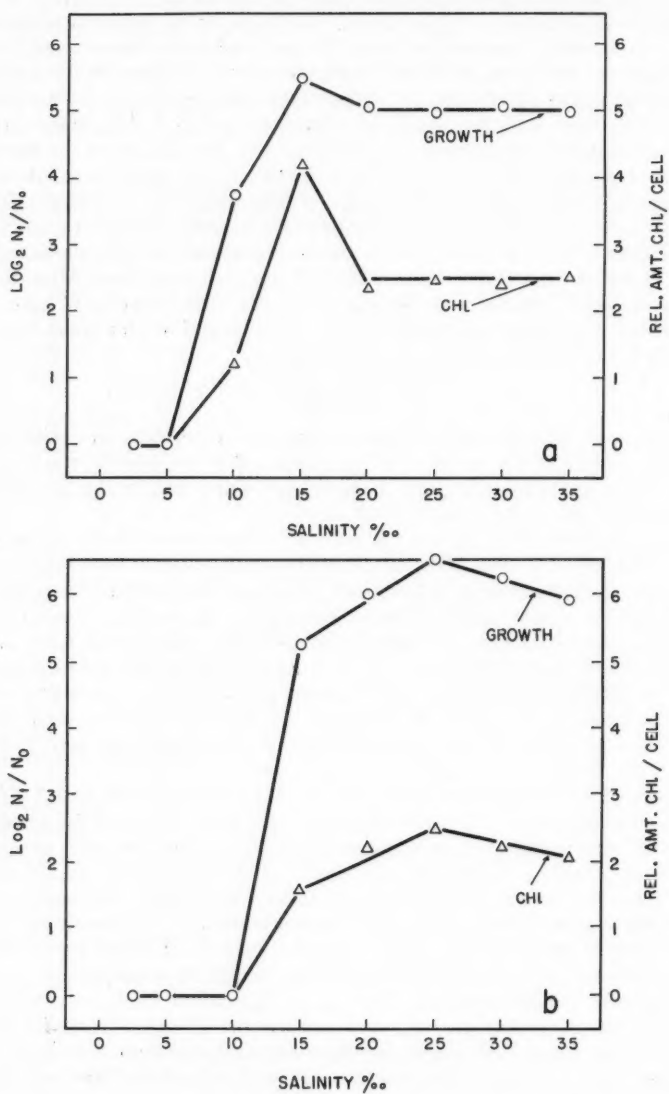


FIG. 1a. Growth and relative amount (see text) of chlorophyll per cell at the various salinities in *Olithodiscus* sp.

FIG. 1b. Growth and relative amount of chlorophyll per cell at the various salinities in *Amphidinium carteri*.

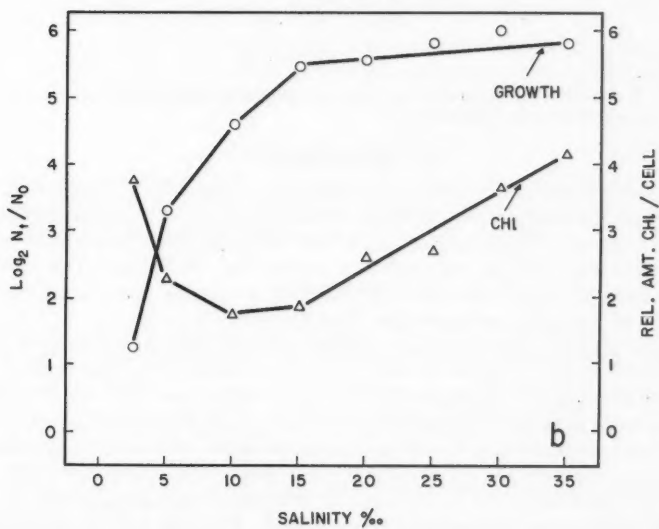
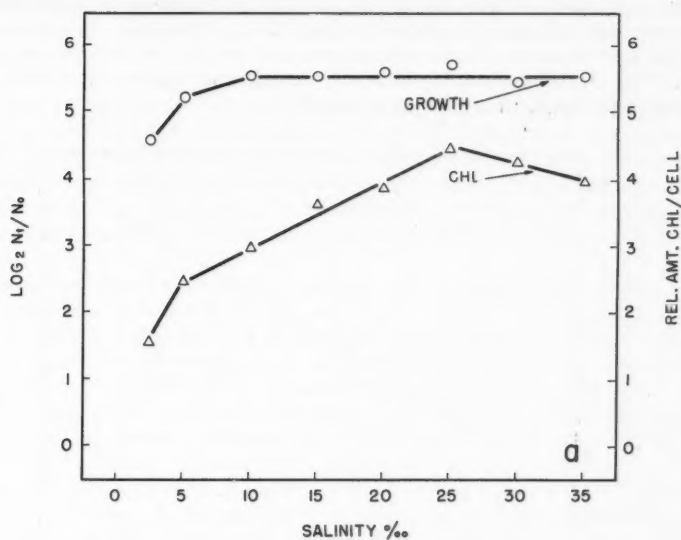


FIG. 2a. Growth and relative amount of chlorophyll per cell at the various salinities in *Porphyridium* sp.

FIG. 2b. Growth and relative amount of chlorophyll per cell at the various salinities in *Platymonas* sp.

concentrations per cell occurred at 2.5 and 35‰. Minimum concentrations of chlorophyll per cell were recorded at 10 to 15‰. However, the chlorophyll concentration per unit volume of culture medium can be directly related to salinity (Fig. 3). These results indicate an optimum salinity for chlorophyll synthesis in this alga to be 35‰ or greater.

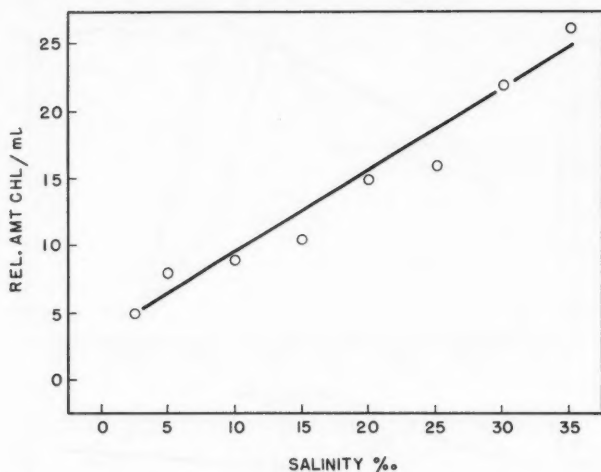


FIG. 3. Relationship between the relative amount of chlorophyll per ml of culture medium and salinity in *Platymonas*.

Discussion

All of the organisms investigated showed a wide salinity tolerance. Two of these algae showed salinity optima; generally there was an optimum salinity range for growth. Where an optimum was demonstrated, this was at a salinity lower than that of full strength sea water (ca. 34–35‰). The chlorophyll results for *Platymonas* do, however, suggest a salinity optimum greater than that of full strength sea water for this organism.

Some organisms are known to be osmotically adaptive and perform little actual work in adjusting to the external environment (10). Most of the osmotic pressure of algal cell sap is due to inorganic salts. The internal concentration of these salts passively changes with the salinity of the environment, and little or no injury occurs (1). In other groups of algae, respiration is affected by changes in salinity (1, 6). Blinks (1) has also cited a case in which photosynthesis was influenced by changes in salinity. Either an increase in respiration or a decrease in photosynthesis means that the supply of compounds produced by photosynthesis and which ordinarily would be available for growth and cellular synthesis is reduced. With multicellular algae, this results in a decrease in size (6), and with unicellular algae there is a decrease in cell concentration. Most of the species investigated in the present study are apparently osmotically labile, but several show a preference for a restricted

salinity range, and hence an increase in growth within this range. It is not known, though, whether this increase in growth is the result of a lower respiratory rate or perhaps an increase in the photosynthetic rate. However, even more direct effects upon the growth process may be involved.

In some organisms, chlorophyll synthesis seems to be affected before cell division within a particular salinity range. *Porphyridium* and *Platymonas* developed a maximum chlorophyll concentration per cell at a certain salinity, although maximum cell concentrations occurred over a broad range of salinities. Contrariwise, *Olisthodiscus* and *Amphidinium* to a lesser extent show the maximum chlorophyll per cell to correspond with the maximum cell concentration.

Platymonas seems to present, in part, an anomalous situation. The maximum concentrations of chlorophyll per cell were obtained at the extremes of the salinity range, the minimum chlorophyll concentrations being at the intermediate salinities. Provasoli *et al.* (12) found that one of their organisms was unable to undergo cell division at unfavorable salinities, but the alga remained viable under these conditions. It would seem that low salinities restrict cell division of *Platymonas* whereas chlorophyll synthesis is not affected to the same degree. There is, however, a direct relationship between the relative chlorophyll concentration per unit volume of culture medium and salinity (Fig. 3). Therefore, at the intermediate salinities, the total chlorophyll content in the culture was greater than at the lower salinities, but was distributed throughout many more cells.

The results obtained in this investigation suggest that salinity is an important factor in controlling the distribution of organisms in the marine environment, especially in areas of low salinity.

Acknowledgments

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A STAINING PROCEDURE FOR THE FLAGELLA OF SOME AGAROLYTIC BACTERIA¹

ARTHUR E. GIRARD² AND ROBERT C. CLEVERDON²

Abstract

The Fisher and Conn staining procedure was used to obtain stains of 10 cultures of marine agarolytic bacteria (tentatively placed in the genus *Alginomonas*). All had a single polar flagellum. Flagella on cells grown in a synthetic medium were easily demonstrated with little stained background. A brief discussion of flagellation as a taxonomic criterion of the agarolytic bacteria is included.

The conventional methods for staining the flagella of marine bacteria were reported as unsatisfactory (4, 8), although Leifson (5) found his procedure to be quite satisfactory for five of our strains. It was postulated that when smears were made with tap or distilled water, the cells become distorted, while in smears with 3% NaCl or sea water, they undergo equally undesirable plasmolytic changes on drying. This report concerns the successful staining of 10 strains, yielding excellent results with minimum effort.

Methods

Test Organisms

The 10 cultures used in this study were isolated from sea water, mud, and algal infusions during a previous study of methods for enumeration of marine bacteria (2). All strains demonstrated a visible depression on agar and have been tentatively identified as members of the genus *Alginomonas*, although they correspond to no described species in Bergey's Manual of Determinative Bacteriology (1). All cultures have been deposited with the College of Fisheries, University of Washington, Seattle 5, Washington, and Dr. J. M. Shewan, Torrey Research Station, Aberdeen. (See Fig. 1 for designations.)

Media

Stock cultures were kept in an artificial sea-water medium (6, 7) consisting of: 0.01% yeast extract; 0.01% $\text{FePO}_4 \cdot \text{H}_2\text{O}$; 0.5% peptone (Gelysate, Baltimore Biological Laboratory); and 2% agar (Difco, Bacto-agar). The stock cultures were transferred monthly and kept at 4° C. The cells were grown without shaking in synthetic media, at 25° C; the diluent for the nutrients was an artificial sea water (6, 7). The pH of all media was electrometrically adjusted to $7.4 \pm .1$. The nutrients for cultures STSB, FJ-7, L-10, L-12, and L-13 were 0.05% L-glutamic acid, 0.1% NH_4Cl , 0.05% galactose; those for L-14, L-15, L-6, and 1ZR1 were 0.15% L-alanine, 0.05% galactose; those for U-1 were 0.2% L-proline, 0.05% galactose. Stains were made when motility was observed in a hanging-drop preparation.

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Preparation of Smears and Staining Procedure

When the organisms demonstrated motility, a loopful was taken from each tube and placed on a tilted slide, the drop being allowed to flow over the surface. (Fresh sonically cleaned slides from the Will Corporation were found to be quite as satisfactory as those cleaned with dichromate - sulphuric acid or with nitric - sulphuric acid mixtures.) The slide was then allowed to air-dry before being stained by the Bailey method as modified by Fisher and Conn (3). The mordant and mordant-dye mixture proved to give better results when allowed to stand at least 1 day before use; fresh mordants, in most instances, were unsatisfactory.

The slide was flooded with solution A with filtering as recommended (3) and was allowed to stand for 3.5 minutes. After A was poured off, without washing, the slide was flooded with B, with filtering, and allowed to remain for 7 minutes, after which it was washed in distilled water. Ziehl-Neelsen carbol fuchsin was then applied for 1 minute while the slide was still wet; the slide was then washed in tap water, and air-dried.

Photography

An American Optical Company research microscope was used, with an achromatic condenser of N. A. 1.40, a 95 \times apochromatic fluorite objective of N.A. 1.25 and a Leitz Mackam attachment. The light source was a Bausch and Lomb microscope lamp with Wratten No. 22 and 58 filters. Critical illumination was used at all times. Kodak Contrast Process Panchromatic film was used at a camera length of 16 inches. Chemicals used for developing and printing were obtained from the Eastman Kodak Company and prints were made on Kodabromide F-2 paper. Some photographs were taken with the Zeiss Ultraphot II, condenser of N. A. 1.40, objective N.A. 1.30; Köhler illumination was used.

Results

As seen in Fig. 1, all the cultures demonstrated a single polar flagellum. The young cultures seemed to have a predominance of spherelike cells such as can be observed in C, D, G, H, and I. As the cultures became older, there was a prevalence of rods exemplified by the cells seen in A, B, E, F, and J. The contact prints, K and L, are included to demonstrate the debris obtained when a peptone medium (K) was used as contrasted to the clean preparation when a synthetic medium is used (L).

Discussion

The flagella of the marine bacteria can be readily stained if the cells are grown in an artificial sea water used a diluent for low concentration of nutrients. A large amount of debris was observed with the use of natural sea water as the diluent, thus obscuring flagella; the same problem occurs when a high concentration of nutrients is used. The major problem of staining flagella of marine bacteria appears not to be fragility of the flagella but rather that inadequate attention has been placed on the medium from which the preparation is made.

No study has been reported of flagellation of the marine agarolytic bacteria. In fact, the flagellation of *Agarbacterium aurantiacum*, type species of genus

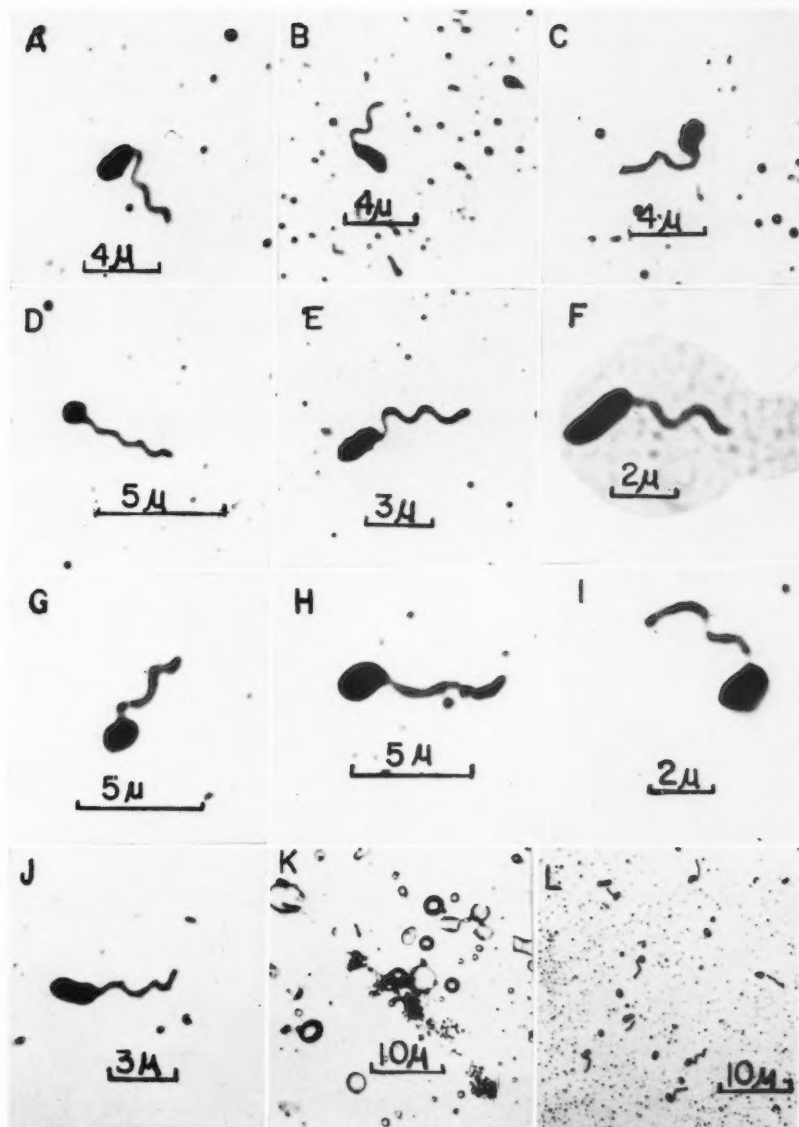
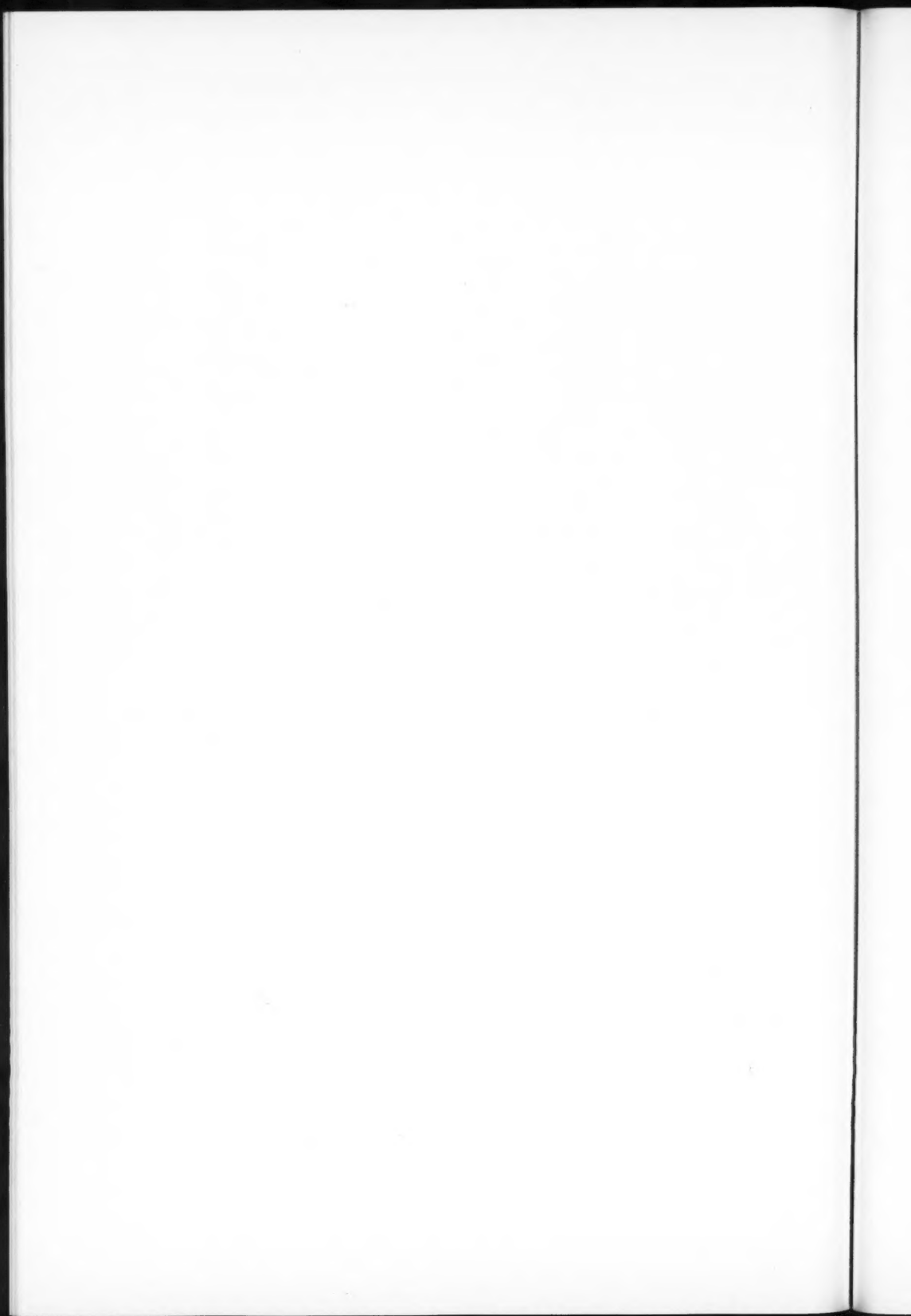


FIG. 1. Photomicrographs of 10 motile agarolytic cultures, showing a single polar flagellum in each case. The culture designations are: A, STSB; B, FJ-7; C, 1ZR1; D, U-1; E, L-6; F, L-10; G, L-12; H, L-13; I, L-14; J, L-15; K, preparation made from peptone medium; L, preparation made from synthetic medium.

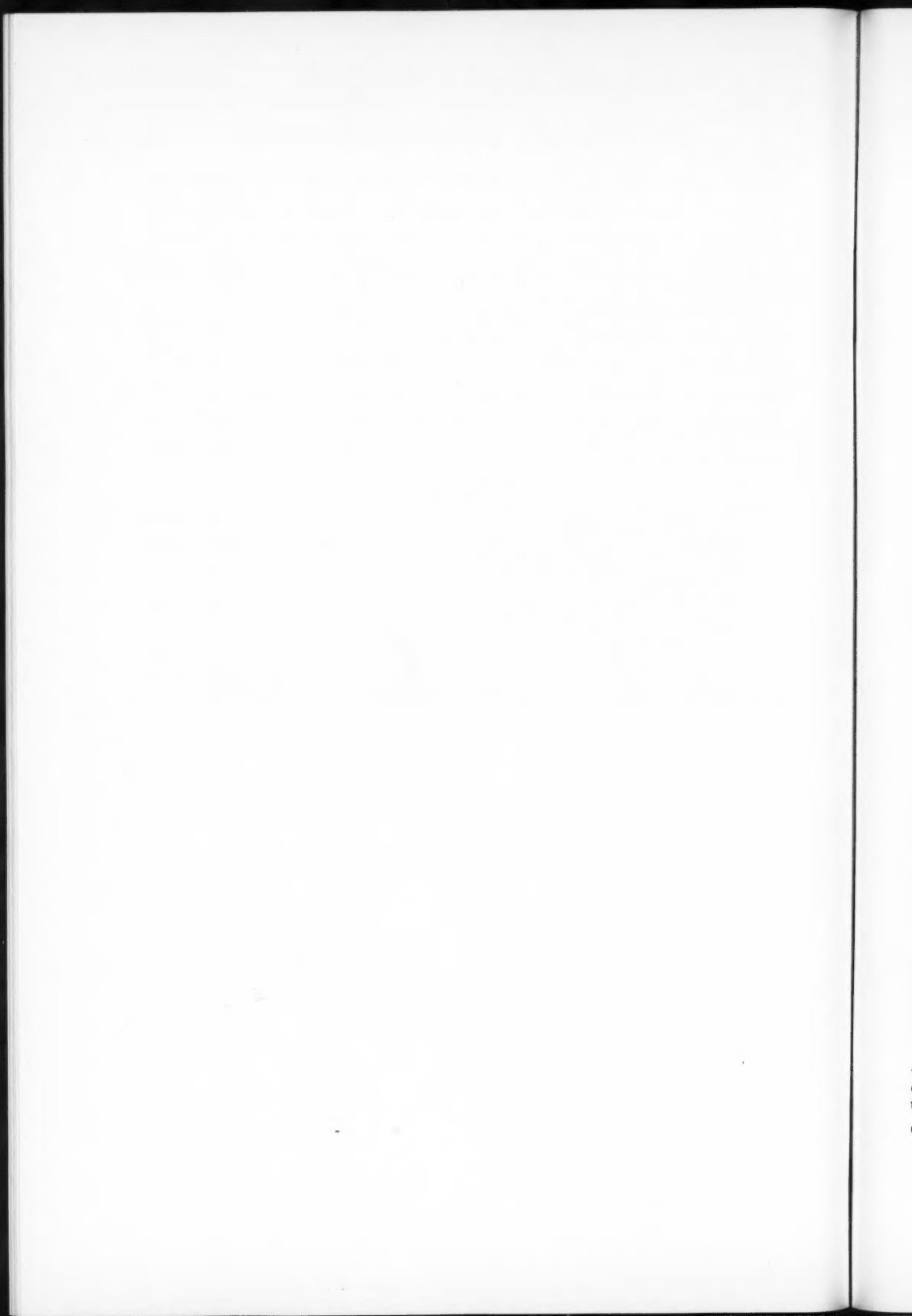


Agarbacterium, which is motile, has not been reported. If this organism was found to be polar flagellate, then the genus *Agarbacterium* would be placed in the family Pseudomonadaceae. The peritrichous, Gram-negative agarolytic species would then remain in the family Achromobacteraceae as at present, constituting either a separate genus or being included as members of others. Flagellation (peritrichous) of only two of the six motile species in genus *Agarbacterium* has been reported.

If genus *Agarbacterium* were placed in the family Pseudomonadaceae, there would result conflict with genus *Alginomonas*, also in this family. Unfortunately the type of flagellation has not been determined for all of the species included in genus *Alginomonas* and it may be found later that some of these species do not belong here. It is evident that the taxonomy of these organisms is equivocal. Studies of the type here reported suggest that so obvious a character as type of flagellation should regularly be reported to facilitate some taxonomic conclusions.

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EFFECT OF ANAEROBIOSIS ON THE EMERGENCE OF STREPTOMYCIN-RESISTANT BACTERIA¹

H. FARKAS-HIMSLEY

Abstract

This paper deals with the increase in numbers of streptomycin-resistant mutants of *V. comma* and *E. coli* B in a so-called streptomycin-sensitive population, when grown under anaerobic conditions. This increase is demonstrated in presence and even in absence of SM. Experiments of a qualitative and quantitative nature are described and a statistical evaluation of the results is given. A relation between the degree of anaerobiosis and SM resistance is demonstrated and the time required for streptomycin resistance to become apparent is shown.

Former observations of SM inactivation under anaerobic conditions are discussed in view of the present findings, and a different interpretation suggested.

Introduction

Inactivation of streptomycin (SM) under anaerobic conditions has been reported at concentrations of SM which were active aerobically (9, 11, 23). Anaerobic conditions were brought about by removal of oxygen or by incorporation into the medium of reducing agents such as thioglycollate, cysteine, or others. SM inactivity was measured in terms of growth, whereas SM activity was measured in terms of growth inhibition. Denkelwater *et al.* (6) used a different approach to determine whether SM is inactivated anaerobically. They stored SM for some time under anaerobic conditions by mixing a reducing agent with the drug. However, on separating the reducing agent they found that SM *per se* was still active.

It seemed to us that these findings, which are contradictory at first sight, might be reconciled. It could be that the drug is indeed not inactivated by anaerobiosis and that the observed growth in the drug's presence was due to the growth of SM-resistant (SM_r) bacteria.

We were further encouraged in this explanation by previously published observations suggesting that SM_r bacteria are in fact geared to anaerobic conditions. Thus, Rosanoff and Sevag (17) found that the metabolic pattern of SM_r bacteria was indicative of anaerobic efficiency. On the other hand it was shown that aerobic growth conditions are unfavorable to SM_r bacteria as compared to their SM-sensitive (SM_s) parent organisms. Thus, it was shown that SM_r bacteria grow more slowly under aerobic conditions than do SM_s bacteria (10, 19) and that their rate of oxygen uptake is slow (8).

The results given in this paper support our suggested explanation since they demonstrate that under *anaerobic* conditions: (a) SM remains active against SM_s bacteria; (b) an increase in the number of SM_r bacteria occurs in the presence and in the absence of SM, which causes a shift in the ratio of SM_r:SM_s in the parent culture.

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Contribution from the Department of Microbiology, School of Hygiene, University of Toronto, Toronto, Ontario. This research was partially supported by a National Research Council Grant, No. A-1008, and a preliminary report of portions of this work was presented at the 10th Annual Meeting of the Canadian Society of Microbiologists, Vancouver, B.C., 1960.

In addition, it was found that SM resistance increased under anaerobic conditions even in the absence of SM.

Materials and Methods

1. Bacteriological

Streptomycin sensitive (SM_s) cultures of *Vibrio comma* A.T.C.C. 9168 and *E. coli* B sensitive to streptomycin (SM_s) were used. The streptomycin resistant (SM_r) mutants were obtained from the SM_s strain by the Lederberg replica plating technique (13) and maintained on agar slants in the absence of streptomycin. Identification of *V. comma* SM_r mutants was based on the following criteria: (a) growth in presence of SM 500 $\mu\text{g/ml}$, (b) Gram staining, and (c) slide agglutination. A SM_r antiserum from rabbits was prepared by two injections, 1 week apart, of 1 ml (approximately 5×10^9) heat-killed SM_r *V. comma*. The antiserum agglutinated SM_r and SM_s bacteria indicating a common antigen in the parent and mutant strain (7). *E. coli* SM_r mutants were identified by (a) and (b).

Inoculum concentration was determined turbidimetrically with a Coleman junior spectrophotometer, and viable counts determined by the pour-plate method (22).

2. Materials

Growth media consisted of (Oxoid) nutrient broth or reduced nutrient broth in which the initial oxygen tension (redox potential or E_h) was lowered as required, by adding varying amounts of reducing agents such as sodium thioglycollate (Difco) or L-cysteine (B.D.H.), or by the removal of air. However, unless otherwise stated, thioglycollate was used. Comparative experiments at different E_h were always adjusted to a constant initial pH.

Streptomycin sulphate (Glaxo) dissolved in distilled water was used.

3. Methods

The E_h was determined by the potentiometric method using the Beckman pH meter, Model G, with a platinum electrode and the calomel half cell as reference electrode, quinhydrone in 0.1 N HCl being used as standard.

The production of anaerobiosis by removal of oxygen was accomplished either by absorption in alkaline pyrogallol (21) or by catalytic combustion with hydrogen in a Brewer's jar.

Sensitivity to SM was determined by the sensi-disk method described by Loo *et al.* and others (14, 18). Thus, 20 ml nutrient agar was poured into petri dishes, incubated overnight, and stored at 4° C for a maximum of 7 days. The number of viable bacteria in 0.5 ml inoculum in each test was always determined and related to standard curves (see below). The diameter of the zone of inhibition around the filter disks was measured in millimeters. Each datum presented is the average of 12 measurements.

Standard curves were plotted, of which Fig. 1 is an example, relating the number of viable organisms at various growth phases to the zone of inhibition (18). By statistical evaluation it was considered that any deviation between experimental and standard figures in excess of ± 0.5 mm was significant.

Growth curves were plotted by determining viable counts and relating these to turbidity readings in the Coleman junior spectrophotometer at 550 $m\mu$.

Quantitative evaluation of SM_r bacteria in a SM_s population was made by the Cavalli-Sforza and Lederberg liquid method (3, 4). The number of the total population was determined by turbidity measurements and by viable counts. Aliquots of 0.5, and 0.25, and 0.125 ml of the bacterial suspension were then diluted in 20 ml of nutrient broth, which was subsequently subdivided equally into 10 test tubes. Incubation overnight at 37° C followed, during which the bacteria reached their maximum concentration, approximately 4.5×10^8 bac-

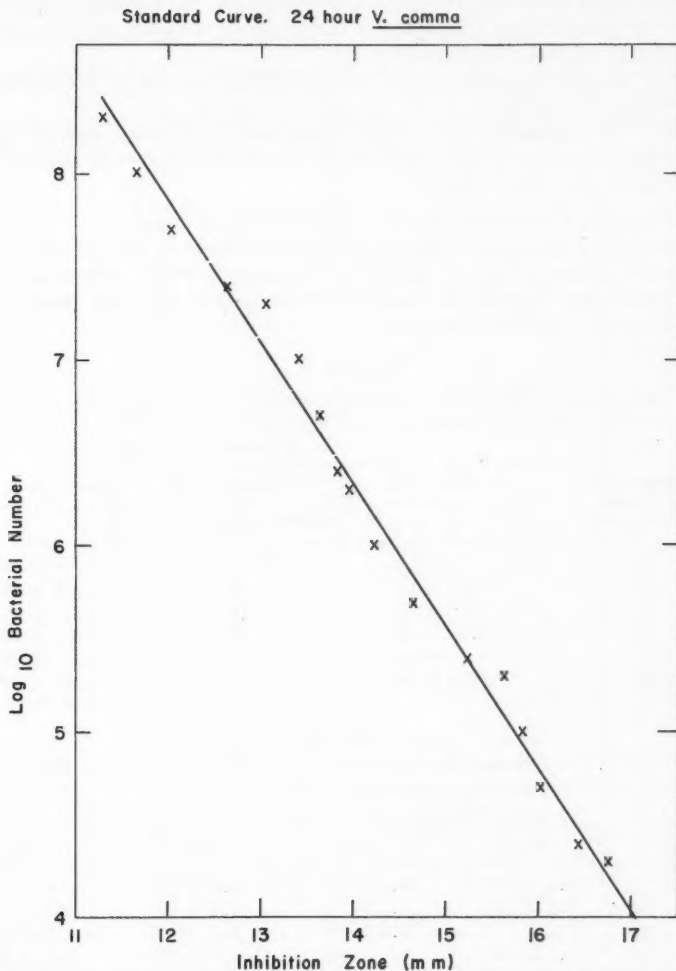


FIG. 1. Example from a set of standard curves relating the zone of inhibition by SM 100 μ g/ml to the numbers in 0.5 ml inoculum and age of SM_s *V. comma*.

teria per ml. The number of SM_r bacteria was determined by spreading the contents of each test tube on two nutrient agar plates containing SM 500 µg/ml. After incubation for 3 to 7 days at 37° C, the number of SM_r colonies was counted. Any modifications in the above procedure are fully described in the text.

Results

Preliminary experiments using *V. comma* showed:

A. That under anaerobic conditions, produced by the various methods outlined above, growth does take place in the presence of those concentrations of SM which are inhibitory aerobically.

B. That the lethal power of SM against SM_s bacteria is not eliminated anaerobically. See Table I.

C. That SM_r mutants of *V. comma* increase in numbers over their SM_s parent organisms when grown anaerobically, whereas the reverse occurs under aerobic conditions. See Table II.

Determination of SM Resistance under Anaerobic Conditions

The bacteria which grew under anaerobic conditions in the presence of SM were tested for their resistance to SM and compared with an identical culture grown aerobically. As control, bacteria grown aerobically and anaerobically in the absence of SM were assayed.

TABLE I
Lethal effect of SM on SM_s *V. comma* in aerobic and anaerobic conditions

Contact time (min) with SM 10 µg/ml	Environmental conditions		
	Aerobe + SM	Anaerobe + SM	Anaerobe - SM
	Viable bacterial count		
0	7×10^8	3.05×10^7	3.05×10^7
15	2.4×10^7	1.1×10^7	3.13×10^7
30	5.6×10^5	2.1×10^6	4.5×10^7
60	2.9×10^4	8.1×10^5	7.4×10^7
120	3.1×10^3	3.7×10^4	1.53×10^8
240	2.45×10^2	$< 10^3$	4.5×10^8

TABLE II
Multiplication of SM_s and SM_r *V. comma* under aerobic and anaerobic conditions

Time (hours)	Aerobic*		Anaerobic†	
	SM _s	SM _r	SM _s	SM _r
0	2.6×10^4	8.9×10^4	2.6×10^4	3.6×10^4
2	6.6×10^4	1.9×10^4	7.0×10^2	5.3×10^3
4		5.4×10^5	3.8×10^4	8.5×10^4
6	3.3×10^8	2.3×10^7	1.4×10^8	4.8×10^6

*Aerobic: growth in broth.

†Anaerobic: growth in reduced broth with 0.03% thioglycollate.

TABLE III
Numbers of SM, *V. comma* in a SM_s population, as influenced by
varying growth conditions

Culture*		Tested against SM $\mu\text{g/ml}$			
		10	25	100	200
		Number of SM resistant bacteria			
Aerobe†	No SM	1	4	1	3
	With SM	Confluent	Approx. 1900	—	—
Anaerobe‡	No SM	Approx. 1800	6	1	6
	With SM	Confluent	Approx. 1900	293	102

*Initial bacterial inoculum $2.7 \times 10^7/\text{ml}$ into 25 ml followed by incubation for 24 hours at 37°C , in absence or in presence of SM $10 \mu\text{g/ml}$. Each culture was then equally divided and plated on SM agar.

†Initial E_h -64 mv.

‡Initial E_h -244 mv.

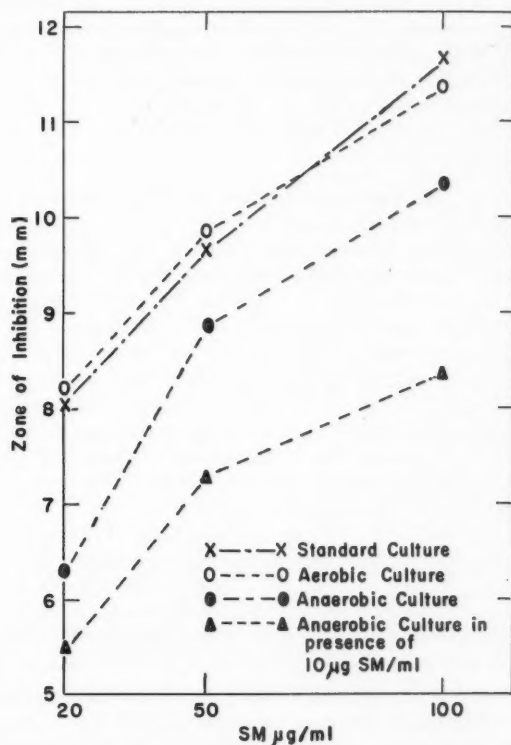


FIG. 2. Determination of SM resistance in SM, *V. comma*, grown for 24 hours at 37°C in aerobic broth and in anaerobic broth, in absence and in presence of $10 \mu\text{g/ml}$ SM. Sensi-disk method was used and referred to a standard curve.

Table III summarizes the number of SM_r bacteria found in 25 ml of the above cultures, after 24 hours' incubation at 37° C. It is evident that SM_r bacteria emerge under anaerobic conditions in the presence and even in the absence of SM. In an aerobic culture exposed to SM their presence in increased numbers is also demonstrated. However, in absence of SM the number of SM_r mutants is much smaller than in the other tests and agrees with that found in any normal SM_s population, i.e., approximately $1:10^9$ SM_s bacteria. (Total bacterial number assayed in the aerobic sample in absence of SM was 3.0×10^9 .)

To confirm further the emergence of SM_r bacteria when grown anaerobically, another approach was undertaken. The SM sensitivity test was applied to populations of known numbers grown aerobically and also anaerobically in the presence and absence of SM. Each sample was assayed for its sensitivity to SM 20, 50, and 100 $\mu\text{g/ml}$ and compared to the inhibition zone produced by similar numbers of SM_s bacteria grown aerobically in the absence of the drug (standard curve). The results given in Fig. 2 indicate that SM_s bacteria grown aerobically have a resistance similar to that obtained for the standard curve. However, bacteria grown anaerobically in the presence of SM show increased resistance toward all SM concentrations tested. Furthermore, it is again noted that bacteria grown anaerobically, in the absence of SM, have an increased resistance. This latter effect, which is also indicated in Table III, warranted further investigation.

Determination of SM Resistance under Anaerobic Conditions in the Absence of SM

Experiments were then carried out anaerobically in the absence of the drug, to determine the growth phase during which increased resistance develops. Control experiments under aerobic conditions were simultaneously performed. Growth curves were plotted and at various time intervals samples were removed and assayed by the SM sensitivity test. Figure 3 shows the results of a typical experiment; a constant drop in the size of the inhibition zone with increase in time and bacterial numbers is clearly seen (see also Fig. 1). However, we find that the anaerobic culture, in spite of having relatively smaller numbers throughout the growth curve (which normally leads to a bigger zone of inhibition), has a smaller inhibition zone than that observed in the aerobic culture at the equivalent growth phase. This indicated a definite increase in resistance for the anaerobic culture.

The SM resistance of the cultures and the time of emergence were determined by reference to the standard curve. Figure 4 shows the difference from standard in both cultures of SM_s *V. comma*. The aerobic culture did not show any increased resistance up to 11 hours, after which a slight increase appeared. This increased resistance was related to the low E_h (-190 mv) which had developed in the medium by this time. The anaerobic culture, however, showed an increased resistance which became apparent after 2 hours' growth, early in the logarithmic phase.

To determine the effect of varying the E_h on the resistance of a population in which drug resistance had been increased by 4 hours' anaerobic growth, bacteria from such a culture were transferred: (a) into aerobic broth, (b) into anaerobic broth, and their drug resistance was tested at intervals.

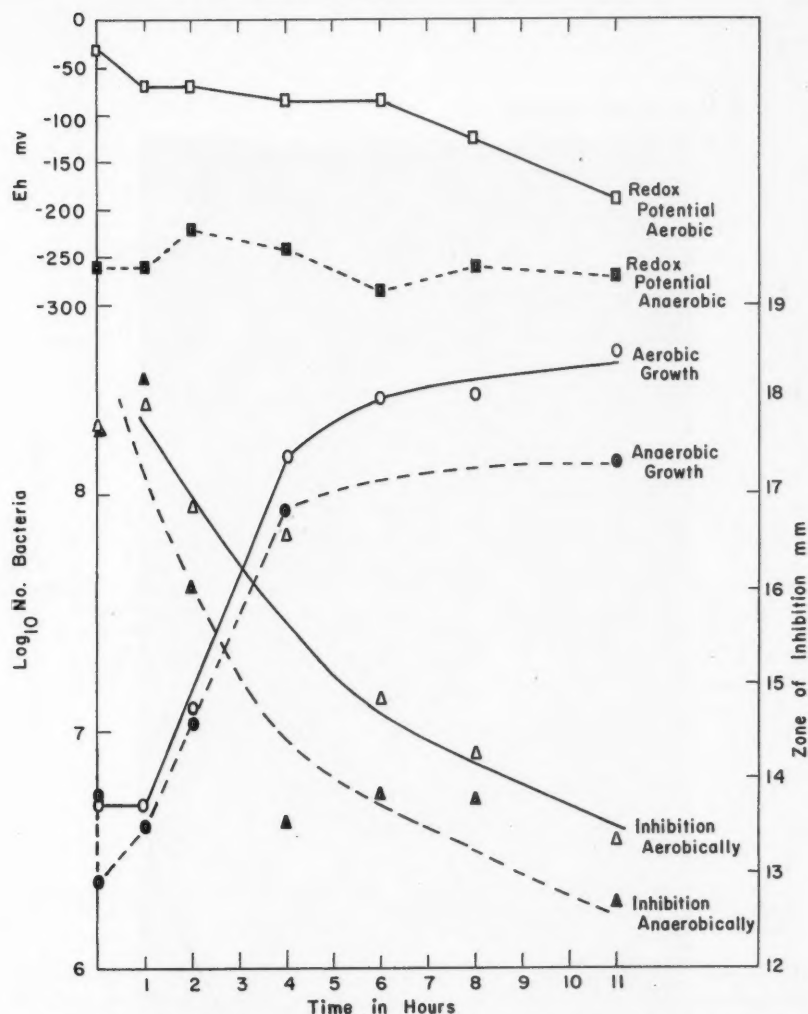


FIG. 3. Relation between growth curve, E_h and increase in SM resistance of $SM_r V. comma$, determined by sensi-disk method using 500 $\mu\text{g/ml}$ SM.

Table IV reveals that under aerobic conditions, significant SM resistance was lost within 4 hours. Under anaerobic conditions, however, SM resistance actually increased for a time and even after 24 hours was still 70% of the initial level.

Next, the influence of various degrees of anaerobiosis on the emergence of $SM_r V. comma$ was determined. Table V shows the relation between E_h and

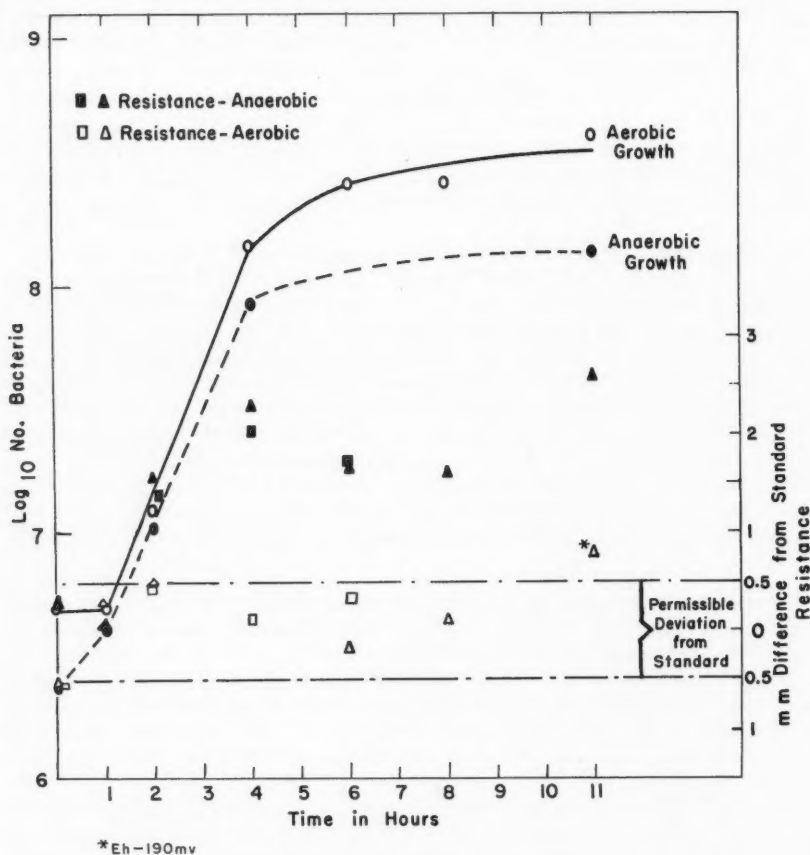


FIG. 4. Determination of SM resistance in SM, *V. comma* during aerobic and anaerobic growth (two experiments each). Sensi-disk method was used with 500 $\mu\text{g}/\text{ml}$ SM. SM resistance is expressed in millimeters of difference from standard curve.

the degree of SM resistance, namely, the lower the E_h (within certain limits), the greater the drug resistance (E_h 's below -260 mv were not tested). Increased SM resistance occurred only at redox potentials of $E_h - 100$ mv and below. In Fig. 5 two photographs demonstrate the relative sensitivities of SM, *V. comma* grown in broth, aerobically and anaerobically. The SM_r bacteria developed anaerobically can be readily discerned in the zone of inhibition, whereas practically no SM_r bacteria are found among bacteria grown aerobically.

In view of these findings a number of quantitative determinations were made. One typical example is given using a modification of the Cavalli-Sforza and Lederberg method as follows:

PLATE I

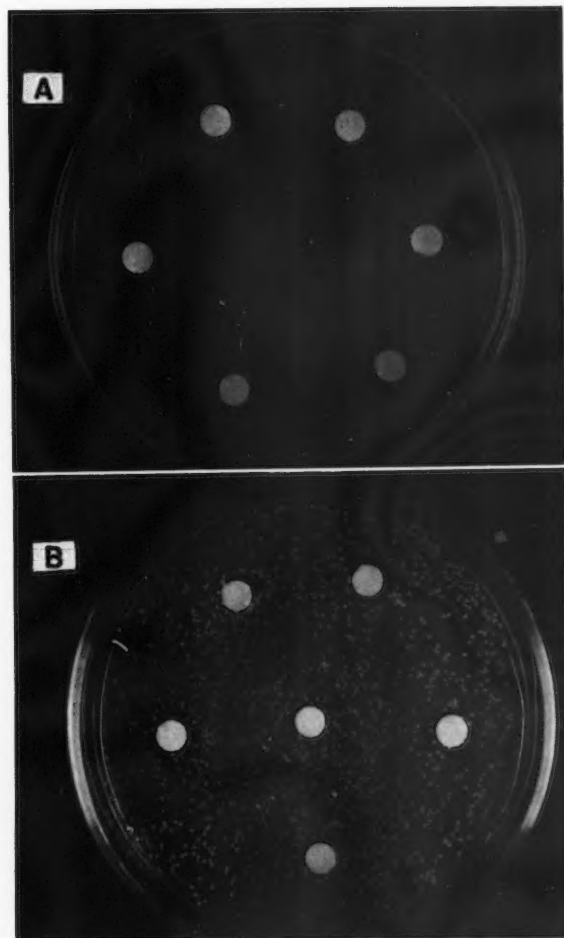


FIG. 5. SM, *V. comma* detected in SM, culture after 3 hours' growth at 37° C and pH 7.3. (A) Aerobically, E_h -54 mv; (B) anaerobically, E_h -232 mv; sensi-disk 500 μ g/ml SM.

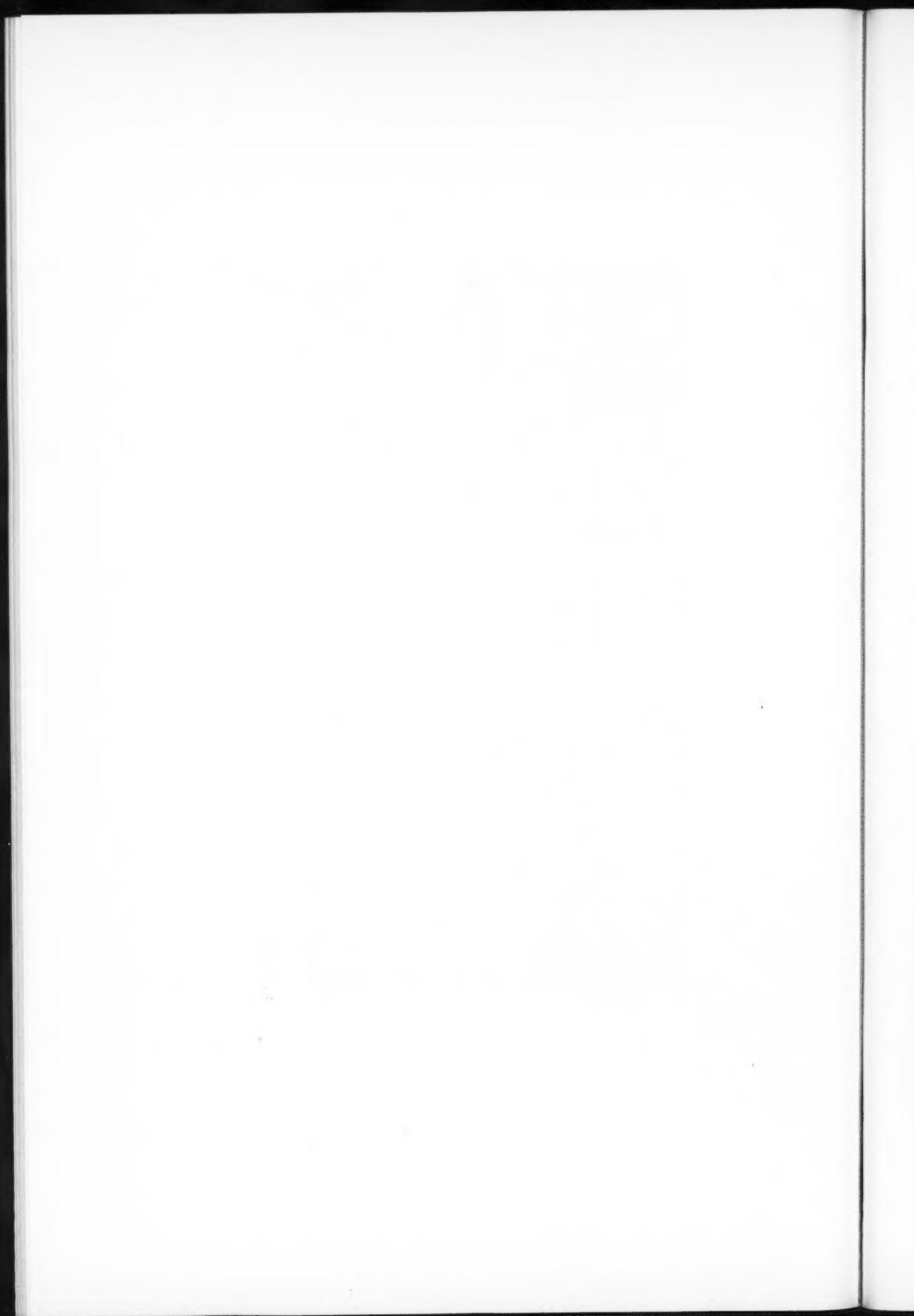


TABLE IV

Influence of E_h on maintenance of SM resistance* in *V. comma*

Growth time (hours)	Deviation from standard (in mm)†	
	Anaerobic, initial E_h -227 mv	Aerobic, initial E_h -70 mv
0	2.00‡	2.25‡
2	2.49	1.33
4	2.24	0.15
6	1.44	—
7	—	0.09
24	1.38	—

*Measured by sensi-disk method using SM 500 µg/ml.

†By statistical evaluation, a difference from standard of 0.5 mm is considered significant.

‡Resistance of a 4-hour anaerobic culture which was used as an inoculum for the above experiments.

TABLE V

Correlation between increase in SM resistance and E_h in *V. comma*

Expt. No.	Initial E_h (mv) in broth	Inhibition zone diameter (mm)		SM resistance,* mm difference from standard
		Experimental	Standard	
1	+20	13.54	13.72	0.18
2	-65	15.04	15.35	0.31
3	-80	17.57	17.70	0.13
4	-100	17.17	18.32	1.15
5	-117	12.46	13.92	1.46
6	-190	12.50	14.25	1.75
7	-233	16.29	18.30	2.01
8	-260	13.55	15.85	2.30

*Measured by sensi-disk method, using SM 500 µg/ml.

NOTE: All cultures were assayed after 3-4 hours' growth.

By statistical evaluation, a difference from standard of 0.5 mm is considered significant.

The culture to be tested under anaerobic conditions was (a) subjected to an "enrichment cycle", i.e., the bacteria were subcultured into reduced broth 3 times in succession, and incubated for 5 hours at 37° C; (b) following subdivision into 10 test tubes for 24 hours' incubation, the culture grown anaerobically was again maintained under reduced conditions. Table VI gives the results which show that the increase in SM_r *V. comma* in a SM_s population grown anaerobically is over 37.5 times that of the culture grown aerobically. By statistical analysis, the average number of SM_r organisms present in the population grown aerobically was 0.53 ± 0.14 and in that grown anaerobically 17.40 ± 1.74 . The marked difference between these values is statistically significant ($P < 0.001$). The number of positive plates of the anaerobic growth is also significantly greater ($\chi^2 = 25.0$; $P < 0.001$). These figures are based on the assumption that an equal number of bacteria was assayed. However, by the viable count method it was determined that a culture of *V. comma* grown anaerobically for 24 hours at 37° C attained only half the density of a culture grown aerobically (see Fig. 4). If equal numbers had been assayed, an even

TABLE VI

Quantitative determination of increase in SM_r *V. comma* under varying growth conditions

No. of SM _r positive					
Aerobic*			Anaerobic†		
Test No.	SM _r positive plates (out of 60)	Bacteria in 3×10^{10}	Test No.	SM _r positive plates (out of 60)	Bacteria in 1.5×10^{10}
1	20	6	4	60	>200
2		6	5		197
3		3	6		166
Ratio	SM _r :SM _s 1: 2×10^9		Ratio	SM _r :SM _s 1: 2.7×10^7	

*5-hour growth in broth; initial E_h -90 mv; pH 7.3.†5-hour growth in reduced broth; initial E_h -230 mv; pH 7.3. Inoculum was subjected to "enrichment cycles" prior to experiment (see text).

higher figure for SM_r grown anaerobically could therefore be expected. The ratio of SM_r:SM_s is thus $1:2 \times 10^9$ in the aerobic culture and $1:2.7 \times 10^7$ in the anaerobic culture, a 75-fold increase in SM_r bacteria grown anaerobically as compared to those grown aerobically.

Discussion

The above data indicate that a reduced environment supports the emergence of SM-resistant bacteria. Therefore, previous reports of bacterial growth under anaerobic conditions in the presence of SM have likely been due in part to an increase in relative numbers of SM_r bacteria in the so-called SM_s "pure" population. Since we have repeatedly observed a relative increase in numbers of SM_r cells in SM_s cultures growing under anaerobic conditions in the *absence* as well as in the presence of SM, obviously drug selection must not be the only mechanism considered in studies of the development of resistance under conditions of low E_h .

The shift towards resistance was expressed within 2 hours of growth in a reduced medium (below E_h -100 mv), when the size of inoculum was about one to ten million cells per ml. It was found that SM resistance was inversely related to the E_h within certain limits, so that by lowering the E_h an increased resistance appeared and vice versa. Once a mixed population increases its resistance, it is liable to modifications by change of E_h in its growth medium. Therefore, if such a population is transferred to a medium with a high E_h (aerobic conditions) it tends to lose its resistance within a few hours of growth therein. However, a single SM-resistant organism isolated from such a population will maintain its resistance even after numerous aerobic transfers on agar in the absence of SM.

Quantitative experiments also confirmed that increased numbers of SM_r bacteria emerge under the influence of anaerobiosis. This may indicate that the small numbers of SM_r bacteria, found in any SM_s population are, when grown anaerobically, under advantageous metabolic conditions. They increase in numbers over the SM_s bacteria, which are depressed metabolically, with a consequent increase in the ratio of SM_r:SM_s.

As these results were obtained with both *V. comma* and *E. coli* B, it seems likely they could apply to other Gram-negative anaerobe-facultative bacteria.

That SM_r bacteria could increase in numbers due to the influence of their growth environment, apart from an effect of drug induction or selection, has been noticed previously both in vitro (20) and in vivo (15). We are not aware that any explanation for these observations has been attempted by the respective authors. In view of our present findings we re-examined the conditions under which increased SM resistance was previously observed in vitro (20) and found indeed a relationship to anaerobiosis, i.e., reduced redox potentials, for cysteine was present in the medium in which an increased number of SM_r mutants was found. As for the in vivo observations by Medlar *et al.* (15), they reported that SM-resistant *tubercle bacilli* were found in increased numbers and of higher degree of resistance in sloughing tuberculous lesions than in unsloughing ones even in patients not receiving any drug therapy. A recent paper (12) shows that unsloughing (open) cavities contain oxygen pressures normal for ordinary and (or) alveolar air; in the sloughing (blocked) cavities, however, the oxygen content is low. Here again our results may explain the in vivo findings of increased resistance to SM under anaerobic conditions.

Current investigations lead us to believe that the metabolic superiority of SM_r bacteria under anaerobic conditions is only a partial answer to their emergence in increased numbers. Evidence is being gathered that SM_r bacteria anaerobically are subject to certain genetic changes. We hope to publish shortly the results of this further work, which will describe a complementary factor for the above phenomena.

Acknowledgments

I am indebted to Professor D. B. W. Reid, of the Department of Epidemiology and Biometrics, School of Hygiene, University of Toronto, for the statistical analysis of the above results. The effective technical assistance of Mrs. P. L. Seyfried is gratefully acknowledged, and the unfailing encouragement given by Dr. A. J. Rhodes is much appreciated.

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NOTES

THE VITAMIN REQUIREMENTS OF *OPHIOBOLUS GRAMINIS* SACC.¹

E. W. B. WARD

White (4) demonstrated that biotin was essential for growth of *Ophiobolus graminis* in synthetic media and that the extent of this growth could be more than doubled by addition of thiamine. Using agar media, Gilpatrick and Henry (1) reached similar conclusions, but Ward and Henry (3), although confirming the requirement for thiamine and biotin, did not find that biotin supported growth in the absence of thiamine. In explanation of the thiamine effect, White suggested that the fungus was able to carry out limited synthesis of thiamine, which in the presence of biotin was sufficient for limited growth. However, as this conclusion was based on total growth as opposed to growth rate data, other interpretations are possible. Thus, similar results might be obtained if the thiamine deficiency was complete and the culture medium was contaminated with a small amount of thiamine. In White's experiments the inoculum consisted of pieces from cultures on potato dextrose agar, and it is possible that the growth which occurred with biotin alone was due to carry-over of thiamine with the inoculum.

Experiments are described below in which vitamin carry-over with inoculum was reduced to a minimum. Both biotin and thiamine were found to be essential for growth, biotin alone being insufficient. In addition, the ability of *O. graminis* to utilize thiazole and pyrimidine as substitutes for thiamine was investigated.

One of the isolates used (Stetson) has been described previously (3), the others were kindly provided by Dr. A. W. Henry of the University of Alberta. The methods employed follow those described by Ward and Colotelo (2). Briefly, the inoculum was prepared by first increasing the mycelium on an organic medium in still culture and using a homogenate of this to seed shake culture flasks containing a vitamin-free synthetic medium. After incubation for 12 days the mycelium from the shake cultures was washed and homogenized, and used to inoculate the experimental media. All homogenates were adjusted to a dry weight of 2 mg per ml, and 1 ml was used to inoculate each culture flask.

The organic medium was composed of malt extract (Difco) 5 g, yeast extract (Difco) 5 g, D-glucose 15 g, and distilled water 1 liter. Still cultures were grown on 25 ml of this medium in 200-ml Erlenmeyer flasks. The synthetic medium contained D-glucose 15.0 g, L-asparagine 2.36 g, KH_2PO_4 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, salts of the following elements in trace amounts, Fe, Zn, Co, Mn, Mo, Cu, Ca; N KOH to adjust the pH to 6.0 and distilled water to make 1 liter. This medium was used both for the growth of inoculum and as a control medium for the determination of vitamin requirements. The vitamins and the

¹Contribution No. 108, Plant Pathology Laboratory, Research Branch, Canada Department of Agriculture, Edmonton, Alberta.

thiamine moieties were included as indicated in Tables I and II. Their concentrations per liter of medium were thiamine HCl 100 μ g, *d*-biotin 10 μ g, thiazole (4-methyl-5-(2-hydroxyethyl) thiazole) 50 μ g, pyrimidine (4-amino-5-aminomethyl-2-methyl pyrimidine.2HCl) 50 μ g. Experiments were carried out using the shake culture method, with 200-ml Erlenmeyer flasks containing 50 ml of medium. Growth was determined as the mean dry weight of four replicate cultures after 10 days' incubation at 15° C.

The data of Table I indicate that for all four isolates negligible growth occurred with either thiamine or biotin alone but that growth was excellent when both were present. It may be concluded, therefore, that for these isolates of *O. graminis* both the thiamine and biotin deficiencies are complete. In the presence of biotin a mixture of thiazole and pyrimidine together supported growth comparable to that with thiamine itself (Table II) indicating that the fungus is able to couple these two moieties to form the thiamine molecule. The lack of growth with thiazole alone indicates that it is unable to synthesize the pyrimidine molecule and the inferior growth and excessive variation with pyrimidine alone suggests that synthesis of thiazole is inadequate for optimal growth.

TABLE I

Effect of thiamine-HCl and *d*-biotin on the growth of four isolates of *Ophiobolus graminis*

Vitamins added	Yield dry mycelium (mg) for isolates:			
	Stetson	II	V	IX
Thiamine-HCl	7.3 \pm 2.0*	5.8 \pm 5.2	4.3 \pm 1.0	4.2 \pm 1.6
<i>d</i> -Biotin	9.7 \pm 3.2	13.9 \pm 9.8	7.1 \pm 2.5	1.2 \pm 1.6
Thiamine-HCl + <i>d</i> -biotin	316.6 \pm 28.6	399.4 \pm 60.4	298.6 \pm 40.1	235.6 \pm 43.4
None	9.5 \pm 3.9	3.2 \pm 4.7	2.1 \pm 1.6	1.2 \pm 1.7

*The mean of four replicates and the sample standard deviation.

TABLE II

Effect of thiazole and pyrimidine on the growth of four isolates of *Ophiobolus graminis*

Vitamins added	Yield dry mycelium (mg) for isolates:			
	Stetson	II	V	IX
<i>d</i> -Biotin + thiamine-HCl	261.7 \pm 46.5*	300.8 \pm 39.9	241.5 \pm 42.0	189.8 \pm 35.8
<i>d</i> -Biotin + thiazole	8.9 \pm 3.6	2.6 \pm 1.3	2.4 \pm 1.7	8.1 \pm 6.3
<i>d</i> -Biotin + pyrimidine	157.4 \pm 26.5	147.1 \pm 63.2	157.1 \pm 82.5	27.6 \pm 2.2
<i>d</i> -Biotin + thiazole + pyrimidine	250.6 \pm 15.9	312.4 \pm 11.6	259.7 \pm 16.8	167.6 \pm 14.1
<i>d</i> -Biotin	7.4 \pm 2.3	4.3 \pm 1.0	12.8 \pm 0.9	3.9 \pm 3.1

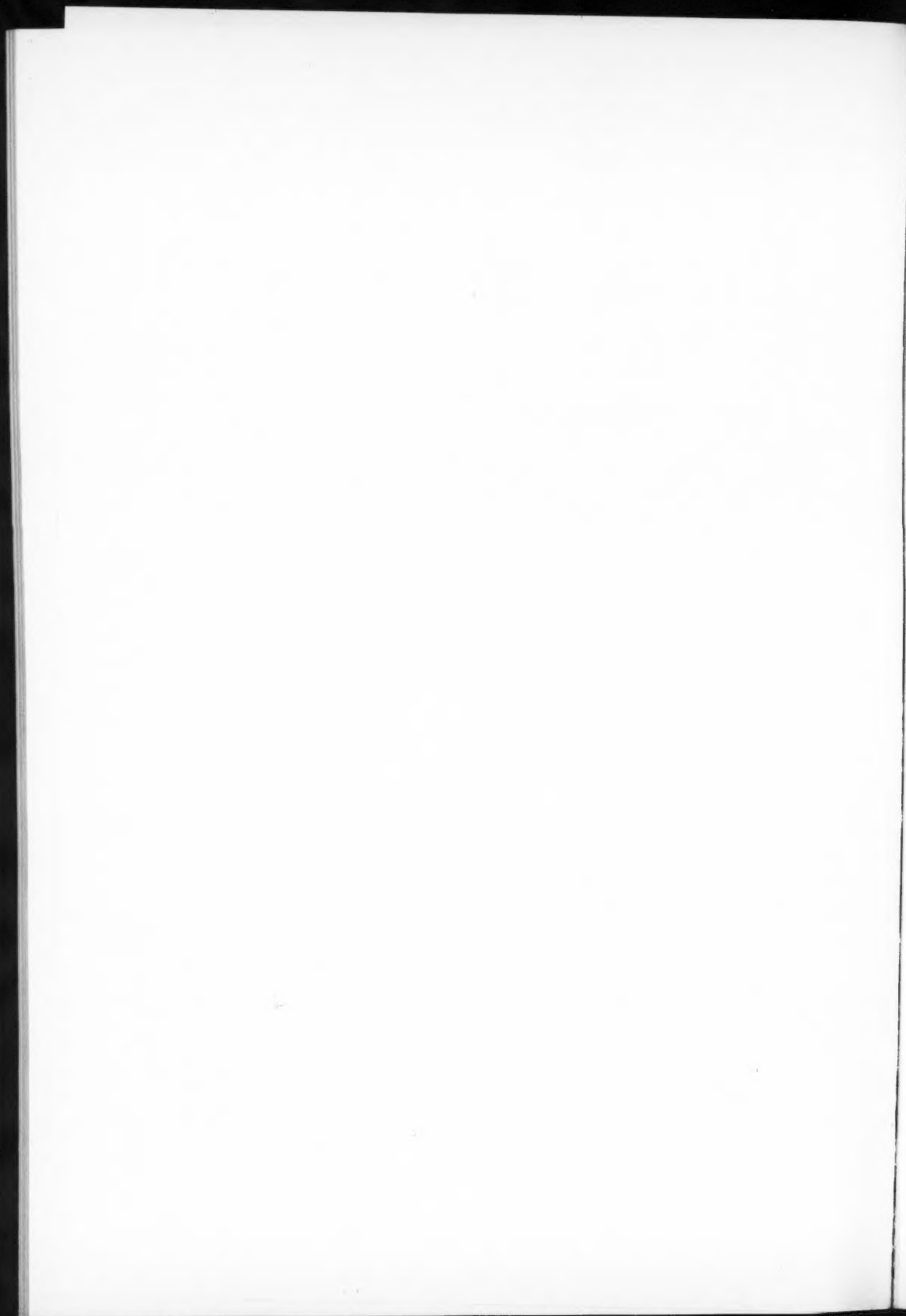
*The mean of four replicates and the sample standard deviation.

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